PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

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STUDIES ON THE NUTRITION AND REPRODUCTION OF PARAMECIUM[1]

(Four figures)

WILLIS H. JOHNSON
University of Chicago, Wabash College, and Stanford University

IN THE last few years the methods of controlling the food in protozoan cultures have been more and more refined. Hargitt and Fray (1917) were the first workers who attempted to raise ciliates on pure cultures of bacteria. They isolated thirty species of bacteria from their hay-infusion cultures and identified eight of them. Using pure cultures of these bacteria in hay infusions, they found that only one of them, *Bacillus subtilis*, would support growth of *Paramecium aurelia* in isolation cultures. These cultures were carried for 12 days. Hargitt and Fray were unable to raise *Paramecium* on pure cultures of *Pseudomonas fluorescens;* but Oehler (1920) found that he could obtain good mass cultures of *Paramecium caudatum*, *P. aurelia*, and *P. putrinum* on pure cultures of *Pseudomonas fluorescens*.

Phillips (1922) used pure cultures of *Bacterium coli*, *Bacillus cereus*, *Proteus vulgaris*, and nine unidentified species of bacteria in hay infusions for the growth of *Paramecium aurelia*. She found that only one of these, an unidentified *Streptothrix*, when used alone would support the growth of *Paramecium*. Philpott (1928) found that *Paramecium caudatum*, *P. aurelia*, and *P. calkinsi* could be

[1] The author wishes to acknowledge his indebtedness to Dr. W. C. Allee for his interest and advice throughout the course of the work.

made immune to the toxic effects of the pathogenic bacterium *Bacillus pyrocyaneus*. He was able to raise *Paramecium* for 100 days on a pure culture of this pathogenic form in hay infusions. He was not able to obtain growth of *Paramecium* using *Bacillus enteritidis*. Chejfec (1929) studied the bacterial requirements of *Paramecium* for the maintenance of life and found that normal division rate of a single animal would not occur in cultures with concentrations of *Bacterium coli* less than 24,000 per cubic millimeter. Glazer and Coria (1930) report that they were able to raise *Paramecium caudatum* on pure cultures of one species of unidentified bacteria. Their attempts to obtain growth with several other kinds failed. Raffel (1930) was able to grow *Paramecium aurelia* for a considerable period using a single species of bacterium, *Bacillus candicans*, and a single species of alga, *Stichococcus bacillaris*, as food. Losina-Losinsky (1931), in studying the feeding reactions of *Paramecium caudatum*, found that bacteria form the chief food substance of *Paramecium* and that some bacteria are taken in in greater numbers than others. *Paramecium* was found to react quite positively to suspensions of *Bacillus subtilis*, less positively to suspensions of *Bacillus fluorescens liquefaciens*, and very slightly to suspensions of *Bacterium coli communae*. The most recent work of this nature on *Paramecium* is that of Phelps (1934). He found that *P. aurelia* would grow well for 50 days on pure cultures of *Erythrobacillus prodigiosus* in a particulate lettuce medium. Reviews of similar studies on other Protozoa may be found in the papers of Luck, Sheets, and Thomas (1931), Johnson (1933), and Phelps (1934).

Myers (1927) studied the course of populations in cultures of *Paramecium caudatum*, using different volumes of hay infusion and different numbers of initial seedings. He found that all cultures raised to a maximum population and then declined. When he used 0.2 cc. of medium, the cultures with one and two animals at the start reached the highest maximum, with the two-animal cultures reaching the maximum first. The four- and eight-animal cultures in the same volume did not reach as high maximum populations. When he used 0.4 cc. of hay infusion, the one-, two-, and four-animal cultures reached about the same maximum populations, with the four-animal culture reaching the maximum first and the

one-animal culture last. The eight-animal culture had a lower maximum population.

The purpose of this investigation has been (1) to study the ability of *Paramecium caudatum* to grow on suspensions of pure cultures of different bacteria in a balanced physiological salt solution and (2) to investigate the course of populations of *Paramecium* in different densities of bacteria.

MATERIALS AND METHODS

The animals used in these experiments were taken from a mass culture of *Paramecium caudatum* which has been kept in this laboratory for the last seven years. A modification of the method described by Parpart (1928) was used in sterilizing the ciliates. In the paired experiments sister-organisms from a pure line were used in each experiment.

The balanced physiological medium which Barker and Taylor (1931) used in raising *Colpoda cucullus* on suspensions of *Pseudomonas fluorescens* and which Johnson (1933) used in growing *Oxytricha fallax* on suspensions of *Pseudomonas fluorescens* was used in these experiments. The concentrated medium was diluted to the approximate concentration of pond water. One cubic centimeter of M/20 NaH_2PO_4 was added to each 30 cc. of this diluted medium for buffering, and the hydrogen-ion concentration was adjusted with small amounts of M/20 NaOH to pH 6.8–7.0, which has been found to be well within the optimal pH range for *Paramecium*.

Cultures of the following kinds of bacteria, which were grown on agar plates, were used: *Escherichia coli*, *Aërobacter aërogenes*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Staphylococcus aureus*, and one unidentified yeast. The bacteria were removed from the plates with a platinum loop and suspended in the medium. The bacteria, taken from a loop which measured 2.5 mm. in diameter and placed in 10 cc. of the medium, formed a suspension which is referred to later as "*x*" concentration. A 5*x* concentration was produced by adding five times the amount of bacteria.

Ground-glass protozoan isolation dishes were used in all of the experiments. Evaporation in the cultures was maintained at a minimum by keeping the dishes in moist chambers. Capillary

pipettes were used in transferring the animals. All glassware used in each experiment was sterilized before it was used. Counts were made with the low power of a binocular microscope. The temperature during these experiments varied from 23° C. to 28° C. Two drops of the medium were equal to 0.1 cc., and 5 drops to 0.25 cc.

EXPERIMENTS WITH DIFFERENT KINDS OF BACTERIA

Eight series of experiments were run using pure suspensions of the six kinds of bacteria, of the yeast, and of the six kinds of bacteria and the yeast mixed together in 2 drops of medium. These experiments were carried for a 10-day period with the temperature controlled at 27° C. Counts were made at 48-hour intervals. Six individuals were isolated singly into each kind of suspension. After each count six individuals were isolated into fresh suspensions of the different kinds. If there were not six individuals left at the time of any count in any series, a sufficient number of paramecia were taken from the stock culture to make up the difference. Table I gives the results of these experiments.

TABLE I

SHOWING GROWTH OF *Paramecium* ON SUSPENSIONS OF DIFFERENT BACTERIA

	2-DAY PERIODS									
	1		2		3		4		5	
	S*	C†	S	C	S	C	S	C	S	C
Yeast	6	12	6	22	6	1	6	24	6	5
Escherichia coli	6	21	6	9	6	8	6	9	6	1
Aërobacter aërogenes	6	14	6	11	6	0	6	13	6	4
Pseudomonas fluorescens	6	12	6	13	6	3	6	18	6	6
Staphylococcus aureus	6	19	6	10	6	3	6	22	6	9
Serratia marcescens	6	24	6	6	6	3	6	11	6	12
Bacillus subtilis	6	52	6	60	6	40	6	52	6	48
Mixed	6	23	6	0	6	18	6	12	6	2

* Starting number. † Number counted.

The 10-day period is divided into five 2-day periods. Six animals were started in each culture, and the numbers found at the end of 48 hours are listed under C. An examination of these results shows that only one of the eight types of suspensions, those of *Bacillus*

subtilis, supported good growth of *Paramecium*. There was some division in all of the cultures, but in no case did the division rate equal that found in the suspensions of *Bacillus subtilis*. Also, there was not a single other type of suspension which supported continuous growth for the 10-day period.

SHOWING GROWTH OF *Paramecium* IN SUSPENSION OF *Bacillus subtilis*

A short experiment was conducted isolating five animals singly into 2 drops of x concentration of *Bacillus subtilis*, with the temperature controlled at 27° C. Counts were made at the end of 24 hours, and five more cultures were started in fresh material. The animals isolated on succeeding days were taken from the preceding cultures. This was done for 6 days, with the following average fission rates: 1.3, 1.2, 1.5, 1.0, 1.0, and 1.4.

TABLE II

SHOWING GROWTH OF *Paramecium* IN 5 DROPS OF *Bacillus subtilis* SUSPENSION

Series	Average Fission Rate	Series	Average Fission Rate	Series	Average Fission Rate
1	2.6	7	2.2	13	2.2
2	1.8	8	1.1	14	3.4
3	1.3	9	1.3	15	2.3
4	1.3	10	2.0	16	1.6
5	1.5	11	1.5	17	1.2
6	1.9	12	1.9	Av.	1.8

Table II gives the results of a 51-day experiment using 5 drops of medium of the same concentration. The cultures in this experiment were carried for 3 days in each case, out of necessity. Each figure represents the average fission rate for the first day of each series of cultures. Here, as in the case mentioned above, each figure represents the average division rate for 1 day of five organisms isolated singly. The temperature in this experiment varied from 25° C. to 28° C.

Obviously, there has been no gradual lowering of the fission rate over this period. These results indicate that *Paramecium caudatum* may be cultured successfully in suspensions of a pure culture of *Bacillus subtilis* in a non-nutritive balanced physiological medium for a 51-day period, at least.

GROWTH OF *Paramecium* IN DIFFERENT DENSITIES OF *Bacillus subtilis*

In the first part of this work nine series of experiments were conducted, each lasting for 3 days. In each series 5 drops of $5x$ concentration of bacteria and 5 drops of x concentration were used. In each concentration five animals were started together and five others

TABLE III

USING $5x$ AND x CONCENTRATIONS OF *Bacillus subtilis*

CULTURES	$5x$ CONCENTRATION			x CONCENTRATION		
	1 Day	2 Days	3 Days	1 Day	2 Days	3 Days
1. Groups	1.0	2.9	3.35	1.2	1.6	1.6
Singles	1.0	2.4	4.03	1.5	2.3	2.4
2. Groups	1.4	3.25	4.38	1.8	3.0	3.2
Singles	1.2	3.0	5.15	2.0	3.45	3.73
3. Groups	1.5	3.15	3.45	1.5	2.1	2.25
Singles	1.1	2.1	3.65	1.3	2.35	3.03
4. Groups	1.6	3.3	3.65	1.7	2.05	2.3
Singles	1.2	3.0	3.9	1.8	2.65	2.8
5. Groups	1.0	2.85	3.58	1.7	2.2	2.55
Singles	1.2	2.55	3.85	1.5	2.6	3.05
6. Groups	1.8	3.4	4.44	2.0	2.4	2.55
Singles	1.1	2.8	5.0	1.9	3.38	3.8
7. Groups	1.6	3.08	3.75	1.6	2.4	2.5
Singles	0.9	2.65	4.85	1.8	3.28	3.53
8. Groups	1.8	3.45	3.8	2.25	2.65	2.65
Singles	2.3	3.4	3.85	2.7	3.1	3.15
9. Groups	1.3	2.85	4.0	2.05	2.75	2.8
Singles	1.3	2.55	4.08	2.2	3.05	3.1

were isolated singly. Counts were made at 24-hour intervals. The temperature in these experiments was that of the room and varied about the same as reported for the experiments of Table II.

Table III gives the results of these experiments. Each figure represents the average fission rate of five animals since the beginning of the experiment. These results show a number of things. At the end of one day in the culture with $5x$ concentration there is no significant difference between the groups and the singles. By the end

of the second day in the $5x$ concentrations the groups are reproducing faster than the singles. The statistical significance here is 0.0026. At the end of the third day the singles have gone ahead of the groups. In this case the statistical significance is 0.0052.

In the x concentrations there is no significant difference between the groups and singles at the end of the first day. But the difference between them during the second and third days is very marked. The singles consistently have a higher rate of reproduction than the groups.

An examination of the groups in the two concentrations shows that the groups in the x concentrations reproduce slightly faster during the first day than the groups in the $5x$ concentrations. The

TABLE IV

5-DAY CULTURES IN $5x$ AND x CONCENTRATIONS

Concentration		Days				
		1	2	3	4	5
$5x$	groups	1.2	3.08	3.87	4.04	3.97
	singles	1.05	2.7	4.58	4.72	5.1
x	groups	1.5	2.3	2.4	2.4	2.23
	singles	1.75	2.87	3.06	3.14	3.17

statistical significance is 0.0108. However, after the first day the groups in the $5x$ concentrations have a consistently higher rate of division.

The singles in the x concentrations reproduce significantly faster during the first day than the singles in the $5x$ concentrations. There is no significant difference between them during the second day, but at the end of the third day the singles in the $5x$ concentration have reproduced markedly faster than those in the x concentration.

With this start on the course of populations of *Paramecium* in different densities of bacteria, it seemed advisable to carry similar experiments for a longer time. Table IV gives the results of similar experiments carried over 5-day periods. This table represents the average of two sets of experiments over a 10-day period. Each figure is the average fission rate of ten individuals. When the results of

the first 3 days in this series are compared with the results given in Table III, the same general relationships are found to exist. During the 5-day period the group culture apparently reached their maximum population, those in the $5x$ concentrations by the end of the fourth day, and those in the x concentrations by the end of the third day. The singles in both concentrations continued to increase during the 5-day period. The graphs in Figures 1 and 2 show the trends of these populations.

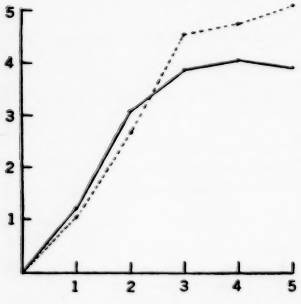

FIG. 1

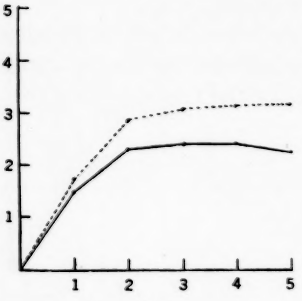

FIG. 2

FIGS. 1 AND 2.—Figure 1 is a graph of the data given in Table IV for the $5x$ concentrations. Figure 2 is a graph of the data in the same table for the x concentrations. In each case the solid line represents the groups and the broken line the singles. Numbers on the ordinates denote the number of fissions, while those on the abscissas denote number of days.

Table V gives the results of a similar group of experiments carried for 7 days. In this case the average numbers of animals produced

TABLE V

7-DAY CULTURES IN $5x$ AND x CONCENTRATIONS

DAYS	NO. OF ANIMALS IN $5x$ CONCENTRATION						NO. OF ANIMALS IN x CONCENTRATION					
	5	1	1	1	1	1	5	1	1	1	1	1
1	17.0	4.3	3.5	3.3	3.0	2.5	21.3	6.8	5.3	5.0	4.8	3.5
2	78.8	13.0	11.0	10.0	11.5	7.3	32.0	10.8	8.5	8.5	8.0	7.0
3	106.0	21.5	18.3	13.8*	15.3†	16.3	32.3	13.5	10.3	10.0	9.5	9.0
4	120.0	31.0	26.3	15.0	20.5	29.9	30.8	11.0	12.0	10.3	9.5	8.3
5	162.5	53.0	41.3	19.0	33.5	45.0	27.0	11.0	12.0	12.3	9.5	8.3
6	147.0	62.0	65.0	31.0	59.3	75.3	25.8	11.0	11.8	9.3	9.0	8.3
7	135.2	69.0	61.0	35.0	70.8	86.3	22.5	11.0	11.8	8.5	8.5	8.3

* One culture died. † Two cultures died.

are given instead of the average fission rates. This table represents the average of four sets of five animals grouped and five animals isolated singly into 5 drops of both concentrations of bacteria. The whole series occupied 28 days. These data plotted in Figures 3 and 4 in terms of average fission rates will, perhaps, more clearly indicate the trends of these populations.

A comparison of the Figures 1 and 3 and 2 and 4 indicates that the same general relationships hold throughout, except that in the 5x concentrations shown in Figure 3 the singles did not surpass the groups during the third day as they had in the previous experiments. They did surpass the groups during the fourth day. The fact that

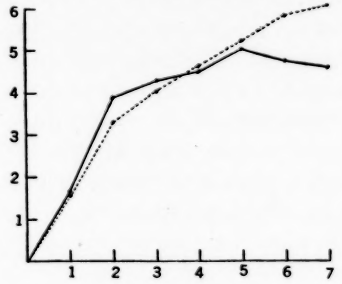

FIG. 3

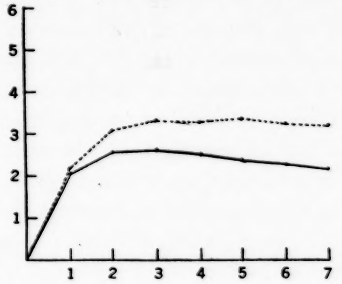

FIG. 4

FIGS. 3 AND 4.—Figure 3 is a graph of the data given in Table V for the 5x concentrations, and Figure 4 is one of the data for the x concentrations. The lines and numbers are used in the same way that they were used in Figures 1 and 2.

three of the twenty single cultures died out during the third day in this series will probably account for this difference.

These studies on population trends in cultures of *Paramecium* show that in the x concentrations the singles have a higher average rate of reproduction for the periods studied, although the difference during the first day is not significant. The highest rate of division for any one day in this density occurs during the first day in both the singles and the groups. The groups reach a maximum during the third day and then begin to decline in fission rate. The singles reach a maximum during the fifth day and then begin to decline.

There is little difference in the rate of reproduction of the groups and singles in 5x concentration for the first day. The highest rate of reproduction for any one day in the 5x concentrations occurs dur-

ing the second day in both the groups and the singles. The groups increase significantly faster than the singles during the second day in this density. During the third day the singles go ahead of the groups and remain so during the periods studied. (The exception to this, shown in Figure 3, seems to be due to the death of three of the cultures.)

Both singles and groups in the x concentrations reproduce faster than those in the $5x$ concentrations during the first day. After the first day the groups in the $5x$ concentration have a much higher rate than those in the x concentrations. After the second day the singles in the $5x$ concentrations have a higher fission rate than those in the less dense cultures.

The data given in Figures 3 and 4 show that all of the populations had reached a maximum and started to decline during the 7-day period except the singles in $5x$ concentrations. Two other experiments carried 8 and 9 days showed that such cultures would reach a maximum population by that time. In no case, however, did any culture starting with a single *Paramecium* reach a maximum as high as those maxima reached by the cultures starting with five animals. An examination of Table V brings this point out clearly.

DISCUSSION

The finding of Hargitt and Fray (1917) that *Paramecium* will grow on a pure culture of *Bacillus subtilis* has been verified in these experiments. Other workers in this field have found that *Paramecium* will live on other kinds of bacteria in pure culture. The literature on this matter seems somewhat conflicting. Hargitt and Fray (1917) were unable to raise *Paramecium* on pure cultures of *Pseudomonas fluorescens*, but Oehler (1920) reported good cultures of *Paramecium* raised on this kind of bacteria. Chejfec (1929) raised *Paramecium* on cultures of *Bacterium coli*, but Phillips (1922) was unable to. In this work *Paramecium* did not grow long on suspensions of *Escherichia coli* or *Pseudomonas fluorescens*. In this connection Hetherington (1934), working with *Colpidium colpoda*, suggests that only particular strains of a species of bacteria may be suitable for a given protozoan. He says, "Indeed, the particular strain of the species used appears to be quite as important as the

larger group. Thus the coli strain from Stanford (*Escherichia coli communae*) and *Aërobacter aërogenes* 5 gave trouble, while *Escherichia communior* 1 and *A. aërogenes* from Stanford were as satisfactory as any bacterium tried."

Hetherington found that *Colpidium colpoda* was "not very particular with regard to the species of bacterium upon which it will grow." Penn (1934) in a discussion of various media suitable for the growth of the Protozoa says, "The question of food of Protozoa in most cases is not so specific. Any nutritive material admissible to the feeding apparatus of the organism concerned is usually satisfactory. The most important factor is the medium." The medium is important. And it appears that some of the smaller ciliates are not very selective in their food requirements. However, from many of the works already mentioned and from these experiments on *Paramecium* it appears that this form is selective in its food and that not any nutritive material admissible to its feeding apparatus is satisfactory. Johnson (1933) found that *Oxytricha fallax* was rather selective in its food.

Hetherington (1934) states that he obtained better cultures of *Colpidium* in nutrient media than he did in a salt solution. Phelps (1934), in his work with *Paramecium*, concluded that some organic matter other than the bacteria was essential in the medium. He found that *Paramecium* grew well on a culture of *Erythrobacillus prodigiosus* in a dried-lettuce medium. But he found that *Paramecium* would not grow on a culture of the same bacteria in a medium made by ashing dried lettuce leaves and dissolving the residue in distilled water. It may be that some kinds of bacteria work better than others as food for particular ciliates in non-nutrient media. Also, Phelps might have obtained different results using some other salt solution. In these experiments *Paramecium* maintained a high fission rate for a 51-day period in suspensions of *Bacillus subtilis* in a non-nutritive medium. Raffel (1930) working with *Paramecium*, Barker and Taylor (1931) working with *Colpoda*, and Johnson (1933) working with *Oxytricha* obtained similar results using non-nutritive media.

The results given in Tables III, IV, and V showing the fission rate of the groups in the 5*x* concentrations higher than the singles

in the same density during the second day are similar to those reported by Johnson (1933) for *Oxytricha* raised on suspensions of *Pseudomonas fluorescens*. These results may be interpreted as due to bacterial crowding. The groups are able to reduce the density of bacteria to a more optimal density faster than the singles, and thus show a higher rate of reproduction.

When the population trends in the different densities over the 3-, 5-, and 7-day periods are compared with those reported by Myers (1927) using hay medium, several differences are noted. The experiments reported here show that, within the limits used, the greater the density of bacteria the higher the maximum population reached with the same initial seeding in the same volume of medium. Myers found that the one- and two-animal cultures in 0.2-cc. hay medium arrived at the same maximum population, while the four-animal cultures had a lower maximum. In this work the one-animal cultures in 0.25 cc. of medium in both densities of bacteria never reached as high maximum populations as the five-animal cultures reached.

The highest rate of reproduction in these experiments occurred during the first and second days. There appeared to be plenty of bacteria in all of the cultures at the end of 7 days, even in the x concentrations. That the decline in the fission rates and the failure of the singles to reach the maxima reached by the groups was not due to inadequate amounts of bacteria seemed certain. It appears that the presence of excretory substances will not explain all of the differences between the groups and the singles. Hetherington (1934) has suggested a possible reason for this difference. He says, "The bacteria removed from an agar plate by means of a platinum needle undoubtedly retain not only small amounts of metabolites but also some of the nutritive substances. This should provide for the continuance of more or less metabolism in Peter's medium." He found that washed *Bacillus niger* supported only a few divisions of *Colpidium;* that, unwashed, this bacterium, although giving no visible signs of metabolism, supported growth of *Colpidium* with a division rate of 2.70; and that the only bacterium clearly showing metabolism in the salt solution gave the highest division rate of any tried. He continues, "These results suggest that the consequences of the

metabolism of bacteria are crucial for the growth of *Colpidium colpoda*."

In view of the facts that the highest fission rates occurred during the first 2 days in these experiments, that there were still plenty of bacteria in the cultures at the end of 7 days, and that the rate of reproduction of the paramecia, even in the single-animal culture, did not increase proportionately after the second day, suggesting some change in the bacteria, it may be that Hetherington's suggestion is the explanation of these differences. Additional experiments must be carried out, using known bacteria in a nutritive medium, before further comparisons can be made between the type of population trends reported by Myers (1927) and those reported here.

SUMMARY

1. Attempts were made to grow *Paramecium caudatum* in suspensions of *Escherichia coli*, *Aërobacter aërogenes*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Serratia marcescens*, *Bacillus subtilis*, and an unknown yeast, and mixtures of all of these, in a non-nutritive salt solution.

2. Suspensions of *Bacillus subtilis* alone supported good growth of *Paramecium*. Over a 51-day period the average daily fission rate was 1.8.

3. Using five-animal and one-animal cultures in both $5x$ and x concentrations of bacteria, both the groups and singles in the x concentration had higher fission rates during the first day.

4. After the first day the groups in the $5x$ concentrations had higher reproductive rates than the groups in the x concentrations.

5. After the second day the singles in the $5x$ concentration had a higher rate of division than those in the x concentrations.

6. The singles in the x concentrations reproduced significantly faster than the groups in the same concentration after the first day for the 7-day period.

7. The groups in the $5x$ concentrations reproduced significantly faster than the singles during the second day. After the third day the singles had a higher fission rate. The difference during the second day is interpreted as being due to bacterial crowding.

8. All of the cultures reached a maximum population during the

7-day period and started to decline, except the singles in the 5x concentrations.

9. The highest rates of fission in all of the cultures occurred during the first and second days.

10. In no case did any of the single-animal cultures reach as high a maximum population as did the group cultures. It is suggested that changes in the bacteria may be responsible for this.

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EFFECTS OF HIGH VACUA AND EXTREME TEMPERATURES ON CYSTS OF COLPODA CUCULLUS[1]

(Five figures)

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DURING the spring and summer of 1931, investigations were undertaken on the effects of extreme temperatures on highly desiccated cysts.[2] Filter paper on which *Colpoda cucullus* had been induced to encyst was exposed to room humidity and temperature for several weeks and then cut into small rectangles. These rectangular pieces were carefully rolled, and to each piece was clamped a U-shaped segment of soft iron wire. Thereupon they were subjected for several days to high vacuum (about 10^{-5} mm. Hg) by means of a mercury pump which was provided with a liquid-air trap and an ionization gauge. Small pyrex tubes were sealed alternately to either side of a large extension tube on the high vacuum side of the mercury pump and kept at high temperature (450° C.) during the 2 or 3 days of evacuation. Into these small tubes, after cooling, the rolled pieces of filter paper were introduced with the aid of a magnet. The tubes were then sealed off.

Fifty-three such tubes which had been tested for high vacuum by means of an induction coil were placed from time to time into liquid air for periods from 1 hour to 12½ days. After exposure, the tubes were broken and the small clamps were removed from the filter paper. These pieces of filter paper were thereupon immersed in balanced medium; and, with no exceptions, free-swimming *Colpoda* were observed in the medium in the course of several hours. Controls, of dishes and medium, were negative.

[1] The major portion of these experimental studies was supported through grants from the National Research Council and the Rockefeller Foundation, to whom we are duly grateful.

[2] Acknowledgment is made to Dr. W. C. Allee, of the Whitman Laboratory, and to Dr. H. B. Lemon, Department of Physics, University of Chicago, for generously providing facilities for the conduct of these preliminary experiments.

Similarly, cysts on filter paper which were air-dried for several weeks, but without evacuation, withstood direct exposure to liquid air. In some instances the cysts on filter paper were introduced directly into the liquid air; in other cases they were subjected by steps to the temperature of melting ice and of CO_2 ice and finally to that of liquid air. Conversely, the temperature for these cysts was raised by the same steps from liquid air to room temperature before inducing excystment. In all of these cases, also, free-swimming *Colpoda* were, in due course, found in the excystment medium.

Tubes containing the cysts on filter paper and sealed off under high vacuum were exposed for $11\frac{1}{2}$ minutes to a temperature of 150° C. From these pieces of filter paper, free-swimming *Colpoda* were likewise afterward recovered, although, owing to the high vacuum in the tubes, it is not certain that the enclosed cysts themselves actually attained that extreme temperature.

These preliminary studies suggested the need of further work with improved methods, as applied especially to the thermal resistivity of cysts of *Colpoda* depending upon: (1) the rate of evacuation, (2) the degree of desiccation, (3) the rate of temperature change, and (4) the period of thermal exposure. The results of these later studies will now be presented.

MATERIALS AND METHODS

When experimentally induced cysts of *Colpoda cucullus* are passing from the wet to the dried state (Taylor and Strickland), the most conspicuous feature is their great shrinkage in volume. Presumably, this is due to a rapid loss of water from the protoplasm and the cyst membranes. Similarly, von Brand (1924) observed that *Vorticella microstoma* loses about 50 per cent of its volume between the spherical maximally contracted free-swimming form and the cyst. The cysts of *Colpoda* which were in contact in the wet state are, in the air-dry state, separated by a space frequently greater than the radius of the dried cysts. The latter usually remain connected, however, by a bridge of the dried glutinous ectocyst.[3]

[3] It may be inferred that this glutinous membrane is comparable with that described and illustrated by Goodey (1913) as the "ectocyst." Weyer (1930) calls a similar structure in encysted *Gastrostyla steinii* the "Gallertartige Hülle" and applies the names "ectocyst" and "entocyst" to the intermediate and innermost membranes, such as may also be found in the cysts of *Colpoda cucullus*. Apparently an unfortunate confusion exists, therefore, in the usage of these terms.

Upon the addition of excystment medium it is but a matter of seconds for the dried cysts to swell to their former size. This rate of swelling is apparently independent of the age of the cysts. The time required for the completion of excystment, however, is increased about tenfold in the first six months, although the viability of the cysts (on Cellophane[4]) is decreased only about 15 per cent during that time (Taylor and Strickland).

The cysts of *Colpoda* may be kept for a very long (as yet undetermined) time in either the wet or dried state. Excystment was consistently induced for these experiments by washing and crowding the *Colpoda* (Barker and Taylor, 1931) by means of the centrifuge. A small drop of the concentrated culture was put into a sterile watch glass, and encystment followed in a few hours. Twenty-four hours later, 1 cc. of sterile balanced medium was added to each dish. If no organic solution is present, the cysts will remain quiescent indefinitely. With proper precautions, more balanced medium can be added as required. It should be noted, however, that an exceedingly small amount of fresh organic solute in such a small quantity of medium may effect partial or even complete excystment. In this paper we refer to cysts kept in this manner as "wet cysts." "Air-dry cysts," on the other hand, may be readily prepared on a Cellophane substrate according to the method described by Taylor and Strickland. By this convenient method, pieces of the Cellophane with a suitable number of dried cysts may be put into watch glasses or into glass tubes and thus subjected to experimental treatment as desired. The inertness and transparency of Cellophane afford an ideal substrate for quantitative, as well as qualitative, studies on the dried cysts of this invaluable ciliate.

Excystment was induced, throughout the following experiments, by a sterile solution of 1 gram of yeast extract paste in 1 liter of balanced salt medium.

For the production of high vacua a mercury diffusion pump and a Wegner-Deka-Micro fore pump[5] were used, finally assisted by a tube

[4] We gratefully acknowledge the generous supply of Cellophane and samples of material supplied by the Du Pont Cellophane Co., Inc., of New York.

[5] This high-vacuum equipment was provided us with the aid of funds contributed by the American Medical Association, for which acknowledgment is hereby gratefully made.

of activated charcoal immersed in liquid air. For convenience in handling in the treatments with high vacua and liquid air, the dry cysts on Cellophane were put into glass tubes of about 2 mm. internal diameter and 15 mm. in length. The one end of each tube was somewhat constricted; the other, lightly plugged with a few threads of cotton.

To determine the effect of heat on dry cysts, a thermostat-controlled electric oven was used. The bulb of the thermometer was in contact with the center of one of the watch glasses. In the case of wet cysts, the watch glass with the bulb of the thermometer in contact with it was completely immersed in water. In all cases the rate of rise in temperature was maintained as nearly as possible at 10° C. every 6 minutes.

EXPERIMENTAL

A. THE EFFECT OF RAPID EVACUATION OF DRIED CYSTS

Pieces from cyst-Cellophane preparations Nos. 371, 372, and 373 were used. The pressure was brought down very rapidly and was held at about 1×10^{-5} atmospheres for 24 hours. Raising the pressure took about 5 minutes. The results are shown in Table I. The

TABLE I

MORTALITY DUE TO RAPID EVACUATION

Preparation No.	Excystment		Mortality (per Cent)
	E_1 Untreated (per Cent)	E_2 Rapid Evacuation (per Cent)	
371	73	39	46.6
372	64	37	42.2
373	68	36	47.1

mortality is expressed $(E_1 - E_2)/E_1 \times 100$. Many shattered cysts, due to the rapid fall of pressure, were visible under the microscope.

B. THE EFFECT OF GRADUAL EVACUATION OF DRIED CYSTS

The pressure was decreased by steps for 4 days until approximately 1×10^{-5} atmospheres were reached, and maintained around that point for 3 days more. The pressure was then slowly raised, by va-

rious devices in the course of a further 3 days, to atmospheric pressure. Table II and Figure 1 show the effect produced on the percentage and rate of excystment. These results show that viability is not affected by gradual evacuation. It is only a coincidence that

TABLE II

THE EFFECT OF GRADUAL EVACUATION OF DRIED CYSTS

AIR-DRY UNTREATED		AIR-DRY EVACUATED	
Time (Hours)	Excystment (per Cent)	Time (Hours)	Excystment (per Cent)
0	0	0	0
2¼	8.6	2	2.6
2¾	38.7	2½	15
3¼	49	3	32
3¾	55	3½	41
4¼	60	4	48
4¾	62	4½	53
5¼	64	4¾	56
5¾	66	5¼	60
20	71	20	71

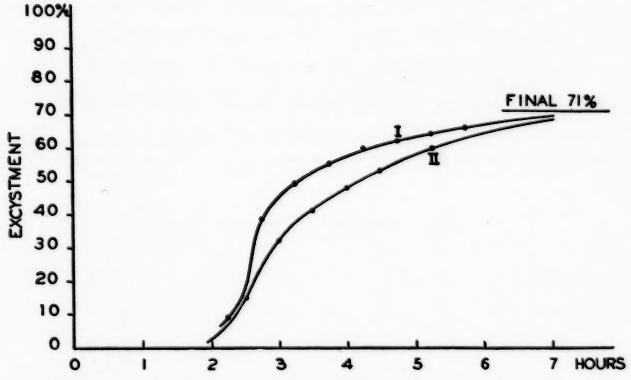

FIG. 1.—The effect of gradual evacuation of dried cysts. *I*, untreated; *II*, evacuated

the untreated and the evacuated cysts gave in this instance the same total excystment (71 per cent). The final count at 20 hours was made many hours after excystment was actually completed. The average final excystment in all (sixteen) tests with the untreated material

was 76.4 per cent; in all (ten) tests with the evacuated material, it was 76.7 per cent.

C. A COMPARATIVE STUDY OF THE EFFECT OF EXPOSURE TO HIGH TEMPERATURE ON WET AND DRY CYSTS

a) *Momentary exposure.*—At certain temperature intervals a watch glass was removed from the oven. Table III and Figures 2

TABLE III

EFFECT OF MOMENTARY EXPOSURE

Wet cysts:												
Temperature (°C.)	34	44	45	49	50.5	51	52					
Excystment (per cent)	100	100	98	94.7	88	78	0					
Dry cysts:												
Temperature (°C.)	35	50	65	75	80	85	90	97	105	110	118	125
Excystment (per cent)	98	97	98	91	94	93	92	97	93	81	66	0

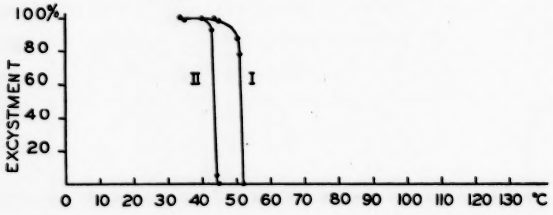

FIG. 2.—Effect of high temperatures on wet cysts. *I*, momentary exposure; *II*, exposure for 1 hour.

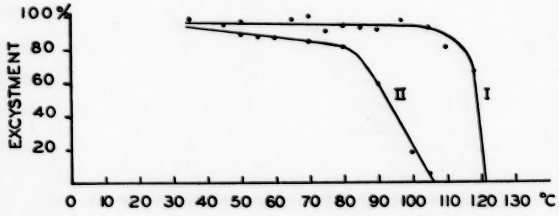

FIG. 3.—Effect of high temperatures on dry cysts. *I*, momentary exposure; *II*, exposure for 1 hour.

and 3 show the results obtained. From Figures 2 and 3 it appears that the lethal temperature for wet cysts is 51° C. and for air-dry cysts 121°. It should be noted, however, that these "air-dry" cysts are

no longer in equilibrium with the water-vapor tension of the laboratory but have become progressively drier as the temperature increased.

b) *Exposure for 1 hour.*—In the case of each watch glass the temperature was raised at the usual rate from laboratory temperature to the desired level, and maintained at that point for 1 hour. Table IV and Figure 3 show the results obtained.

TABLE IV

EFFECT OF EXPOSURE FOR 1 HOUR

Wet cysts:								
Temperature (°C.)...	35	40	43	44	45	50	60	
Excystment (per cent)	98.6	100	93	5.8	0	0	0	
Dry cysts:								
Temperature (°C.)...	50	60	70	80	90	100	105	110
Excystment (per cent)	89	86.4	85.6	82	59	18	5.5	0

As indicated by Figures 2 and 3 it seems that the lethal temperatures for exposure for 1 hour are for wet cysts 44°; for dry cysts, 106° C. In this case also, the "air-dry" cysts became drier, owing to the rising temperature and the duration of exposure.

D. THE EFFECT OF PROLONGED EXPOSURE TO 70° C.

Some pieces of cyst-Cellophane preparation No. 607 were put into a sterile Petri dish. Similar pieces, each with about five hundred cysts, were put into watch glasses. The temperature of the oven was raised at the usual rate to 70° C. and maintained continuously at that point for 26 hours. At intervals a watch glass was removed. After 8 hours the Petri dish was removed. Table V and Figure 4

TABLE V

EFFECT OF PROLONGED EXPOSURE TO 70° C.

Time (in hours).....	1	2	3	4	5	6	7	8	10½
Excystment (per cent).....	85.4	80.6	62.2	67.4	66.7	69.7	60.0	80	72
Time (in hours).....	12	13½	15	16½	18	20	22	24	26
Excystment (per cent).....	72.5	90	72.5	67	74.5	98.5	90.4	80	73

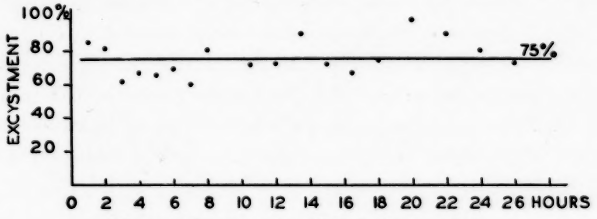

FIG. 4.—Exposure to 70° C. for long period

show the excystment obtained from the cysts in each watch glass.

In order to find out whether absolutely sterile *Colpoda* in large numbers could be obtained in this way, the pieces in the Petri dish were tested for aërobic and anaërobic bacteria and were found to be sterile.

E. THE EFFECT ON DRY CYSTS OF PROLONGED IMMERSION IN LIQUID AIR

Preparation No. 607 was used. The liquid air evaporated continuously during the experiment, so that the temperature was pre-

TABLE VI

EFFECT OF PROLONGED IMMERSION IN LIQUID AIR

Time (in hours)	1	2	4	6	8	11½	13½
Excystment (per cent)	88	79.3	61.3	69	77.8	84.5	81.2

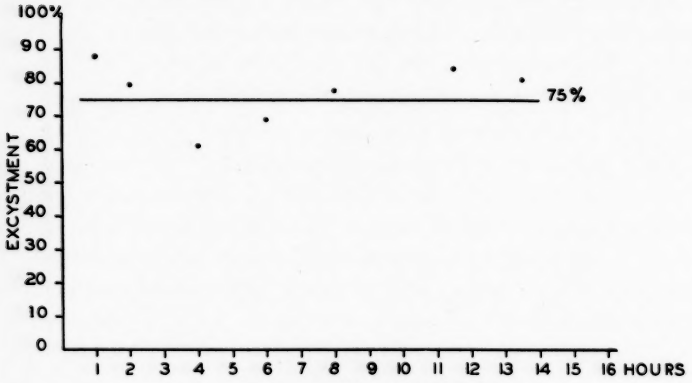

FIG. 5.—Effect of prolonged immersion in liquid air

sumably around −180° C. The capsules were dropped into the liquid air. At intervals a capsule was removed; in about 2 minutes it and the cysts in it had reached the temperature of the laboratory. Table VI and Figure 5 show the excystment obtained. The average excystment was approximately normal for that preparation at the date of these experiments. From the results noted in Table VI, it is evident that these exposures to the temperature of liquid air had no significant effect upon the percentage and rate of subsequently induced excystment.

DISCUSSION

For certain experimental work it would be most valuable to have protoplasm without free (i.e., unbound) water. It seems probable that a high vacuum would be a convenient and effective means toward this end, even though the actual degree of desiccation (water-vapor tension of the protoplasm) cannot be accurately determined.

It is evident from the results obtained by rapid evacuation that protoplasm in equilibrium with the humidity of the laboratory has still much free moisture in it. Later experiments have shown that the rapid rise to atmospheric pressure does not have any injurious effect.

Gradual evacuation does not affect the viability of the protoplasm; but, presumably correlated with increased desiccation, the rate of excystment is at first less for the evacuated than for the untreated cysts. For the example shown in Figure 1, at time 2 hours and 30 minutes the ratio is 1:1.87. Goodey (1913) found that, if cysts of *Colpoda* are kept dried on filter paper for some weeks, their power of excysting rapidly is considerably diminished, though he has no quantitative data. He says, however, that in his tests on the effect of temperature (20°–40° C.) on excystment, at 20° C. a few were active after 2 hours, 12 minutes. This agrees with the curve in Figure 1 for which the temperature is 20°–22° C. and the age of the cyst preparation 36 days.

When the cysts of *Colpoda cucullus* are passing from the wet to the air-dry state, the loss of water is considerable and very rapid. If it is assumed that the temperature of coagulation of a protein in colloidal solution varies with the amount of free water, a comparison of these critical temperatures for wet and air-dry cysts should give some idea of the relative desiccation of the protoplasm. Barker (1933) determined the temperature of denaturation of crystallizable egg albumin at various relative humidities in equilibrium with various water-vapor pressures. Our dry cysts, at the start of the experiment in equilibrium with the humidity of the laboratory, gave 50 per cent excystment after 1 hour at 92° C. Assuming that Barker's results may be reasonably compared with ours, and that an excystment of 50 per cent of the normally viable cysts corresponds

approximately to the mid-point of the denaturation temperature range of albumin, we find from Barker's Table II that the relative humidity should be 58 per cent. The humidity of the laboratory was not measured at the time of our experiments, but this value (58 per cent) for the laboratory seems reasonable enough.

Experiments to determine the effect of high temperatures on the dried cysts in equilibrium with constant and definite water-vapor pressures are now in progress.

For wet cysts the lethal temperature, corresponding possibly to the coagulation temperature of the whole or of some important constituent of the protoplasm at that degree of hydration, is 51.1° C. Goodey states that cystase, the enzyme which he says dissolves the cyst membrane to release the protozoon, is inactivated at 40° C.; but we find that at this temperature, even after 1 hour, excystment seems to be 100 per cent. It may be that, if an enzyme is required to assist the violent exertions of the protozoon to rupture the endocyst, the enzyme is produced only in its heat-sensitive form at the time it is required. He finds that there is no excystment at 40° C. but does not say how long the cysts were subjected to this temperature. It seems probable that it was for several hours. For a period of 1 hour the lethal temperature seems to be about 44° C.

Air-dried cysts on a Cellophane substrate have evidently lost a large part of their free water, as the protoplasm is almost unaffected at 110° C. and may even survive progressive heating to and momentary exposure to 120°. About 20 per cent of the dry cysts can stand the boiling-point of water for an hour. The slope of the curve for 1 hour's exposure (Fig. 3) is an indication of the progressive desiccation of the dry cysts with time and rising temperature.

In this connection it is interesting to note that in 1865 Meunier, "to throw new light on certain cases of spontaneous generation claimed to have occurred in boiled infusions," boiled some hay dust containing cysts of *Colpoda* and found that they did not survive the experience. Dawson and Hewitt (1931) induced excystment of *Colpoda* from dry hay dust after 5 years and 4 months. The time required for excystment was 18–24 hours.

From Figure 4 it is evident that exposure to a temperature of 70° C. for 26 hours does not injure the cysts. After 8 hours' heating,

the cysts in the Petri dish were sterile. No precautions had been taken to protect preparation No. 607 from contamination. It had been handled, cut with unsterilized instruments, and frequently exposed to the air. For some experimental purposes it is important to have absolutely sterile protozoa. It is claimed not that a cyst preparation can be sterilized in this manner under all circumstances but that this preparation, from a culture fed with a non-spore-forming bacterium, was completely sterile after heating as described.

Luck and Sheets (1931) made extensive efforts to sterilize cysts by chemical reagents but without success. Oehler (1924) also tried chemical means of sterilization without success. He was able to obtain sterile *Colpoda* from cysts, dried presumably in the detritus of the medium, which were kept at 37° C. for 6 weeks. He was also able to sterilize cysts, which he had raised in a pure culture of *Saccharomyces exiguus*, by heating them for 24 hours at 64° C.

From Section E it appears that prolonged exposure to intense cold has no injurious effect. The average excystment after $13\frac{1}{2}$ hours in liquid air is 78 per cent, which is only about 4 per cent less than the expected average excystment of the untreated preparation at that date and is within the range of variations expected. Excystment was complete after 8 hours, which is little, if any, more than is required for the untreated cysts (65 days dry). On immersion the cysts must have been frozen instantly. Nor does it seem that a rise in temperature of about 200° in a few minutes has any injurious effect. It is known that sudden freezing with intense cold causes the formation of smaller crystals, consequently with less tendency to disrupt tissues on freezing or thawing.

It would appear that the protoplasm adjusts its water content, more or less rapidly according to circumstances, to the humidity of the environment; and that the effect of such external conditions as high vacua and extreme temperatures on the viability of the protoplasm may be correlated with the degree of its desiccation.

SUMMARY

Experiments on the viability of wet and dried cysts of *Colpoda cucullus* under various conditions are described:

1. The mortality due to very rapid evacuation under the conditions of the experiment was found to be about 46 per cent.

2. The effect of gradual evacuation on viability was found to be nil; there was a noticeable decrease in the rate of excystment.

3. The effect of high temperatures on wet and dry cysts is shown, and the lethal temperatures determined for momentary exposure and exposure for 1 hour.

4. The viability of the cysts is not impaired by exposure to 70° C. for 26 hours, and the cyst-cellophane preparation under test was completely sterile after 8 hours.

5. Intense cold for long periods, such as immersion in liquid air for 13½ hours, does not affect the viability of the cysts; some excystment (percentage unknown) was obtained from cysts exposed to liquid-air temperature for 12½ days.

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THE EFFECT OF DESICCATION ON SURVIVAL AND METAMORPHOSIS OF THE JAPANESE BEETLE (POPILLIA JAPONICA NEWMAN)

(One figure)

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INSECTS manifest considerable variation in their ability to survive desiccation. Breitenbecher (1918) reported that the June beetle, *Lachnosterna*, could not survive a loss by desiccation of 15 per cent of its original weight; whereas, the beetle *Cotalpa lanigera* could survive a loss of 25 per cent, and the potato beetle, *Leptinotarsa decemlineata*, more than 50 per cent.

Insects which live in a comparatively dry medium and feed on dry food seem to possess the capacity to conserve body water. In many of these insects, desiccation may result in a loss of considerable body weight before any change in water content is noted. Thus, Berger (1907) showed that the larvae of *Tenebrio molitor* could be kept in an absolutely dry medium and that, in spite of a great loss of water, the relative fluid content of the body remained approximately constant. Similar results were obtained by Hall (1922) and by Buxton (1930) with *Tenebrio* larvae. The former found that these larvae could be desiccated to 52.6 per cent of their weight without losing vitality; and the latter, that they maintained their original ratio of water to dry matter during a month of starvation at humidities ranging from 0 to 60 per cent. This ability of *Tenebrio* larvae to maintain a constant water content during periods of desiccation is probably due to the utilization of metabolic water, as has been shown by Mellanby (1932*a*). Speicher (1931) placed the second- and third-instar larvae of the Mediterranean flour moth, *Ephestia kuehniella*, in jars containing anhydrous $CaCl_2$ and fed them meal previously dried at 103° C. Despite the dry atmosphere, the larvae and pupae maintained a constant percentage of free water in their bodies as long as they survived. Buxton (1932) observed that the bug *Rhodnius prolixus* lost dry weight and water in the same proportion when kept in dry air even when a weight loss of 50 per cent had resulted.

On the other hand, the larva of the Japanese beetle normally lives in moist soil, where it usually is not subjected to the drying effects of the atmosphere. Therefore a mechanism for the conservation of water is probably not necessary, and this larva might be expected to react to desiccation in a manner different from that of insects living in dry air. For this reason it was thought desirable to determine the rate and manner of desiccation, and the vital limits of desiccation, of the various stages in the life-cycle of this beetle.

Ludwig (1931) found that during the normal metamorphosis of the Japanese beetle from larva to adult there is a loss of weight amounting to one-half of the maximum larval weight, and that during the emergence of the adult the loss of weight amounts to one-third of the pupal weight. The water content also was found to decrease from 78.0 per cent in the larva to 66.6 per cent in the adult. Hence, this weight loss is due largely to a loss of water. Since dehydration normally occurs during the transformation from larva to adult, the question arises as to whether it is merely incidental or is actually essential to the metamorphic changes. Experiments were therefore performed to determine the effects of partial desiccation on the loss of weight and water occurring during metamorphosis.

MATERIAL AND METHODS

The Japanese beetles used in these experiments were obtained from two sources. (1) The third-instar larvae were collected in the field during the winter and spring months and were secured from Dr. Henry Fox, of the Japanese Beetle Laboratory, Moorestown, New Jersey. Prepupae and pupae were obtained from these larvae. (2) The first- and second-instar larvae developed from eggs collected in the laboratory. Each individual was kept in a 1-ounce metal salve box containing moist plant mold. The humidity of the plant mold was kept near the point of saturation by the addition of tap water. Each larva was also given several grains of wheat to serve as additional food. Throughout the feeding period, an abundance of food was always available, except during the period of desiccation, when the larvae were starved.

Desiccation experiments were performed on the first-, second-, and third-instar larvae, early prepupae, late prepupae, and early pupae.

The early prepupa differs from the third-instar larva only in the creamy-white color of its body, due to the elimination of fecal material. In order to obtain comparable results, desiccation experiments were always begun on the first day the individual was definitely recognized as being a prepupa. The late prepupa is distinguished by the degeneration of the legs and mouth parts and the shrinking of the body within the larval skin. Desiccation experiments on the pupae were always started the first or second day after pupation.

In all cases the beetles were kept at room temperature (18°–25° C.) until used in the desiccation experiments. Two groups of experiments were performed. In the first group, a relatively slow desiccation was obtained by placing each beetle in a small, unstoppered vial kept in an incubator at approximately 25° C. First-, second-, and third-instar larvae, early prepupae, late prepupae, and early pupae were desiccated in this manner. During the course of preliminary experiments, it was found that larvae lost weight much more rapidly when placed in metal salve boxes than when placed in glass vials. Consequently, a second group of experiments was devised in which a more rapid desiccation of third-instar larvae was obtained by placing each individual in an uncovered, 1-ounce metal salve box in an incubator at 25° C. The relative humidity of the incubator usually ranged from 30 to 35 per cent. Weight readings were made daily; and when desiccation to the desired degree was obtained, the beetles were returned to the salve boxes containing moist plant mold and kept at 25° C. for further observations. Fecal material was always weighed along with the larva. Daily observations were made on all desiccated beetles, and weight readings were made on the larvae at least twice a week. An individual was considered to have recovered when it had regained its original weight or when it had transformed normally to the succeeding stage. Following desiccation, weight readings were made on each individual when it had reached the following stages: late prepupa; early pupa; and adult, within 24 hours of emergence. All weighings were made on a chainomatic balance sensitive to 0.1 mg.

Readings on the water content of 35 normal and 33 partially desiccated third-instar larvae were made to determine whether all of the loss of weight was due to the evaporation of water. Since this proved

to be the case, determinations of water content of normal individuals in the early prepupal, late prepupal, and early pupal stages were made for the purpose of calculating the water content of individuals when they had reached the maximum fatal limits of desiccation. Water content of adults, obtained from prepupae and pupae which had been previously desiccated, was determined for comparison with similar readings obtained on adults which had not been previously desiccated. To determine water content, each individual was first weighed, then dehydrated in an oven at approximately 60° C. until its weight remained constant, and finally weighed again. The difference between the two readings is interpreted as the weight of the water lost by evaporation.

OBSERVATIONS

Within each stage of the life-cycle there is a tremendous variation in the rate at which desiccation occurs. For instance, first-instar larva No. 24 became desiccated to 51.6 per cent of its original weight in 23 hours, while first-instar larva No. 12 required 142 hours to become desiccated to 54.1 per cent of its original weight. These two larvae were dehydrated at the same time, so that conditions of temperature and humidity were identical. Similar variations were found in each stage of development. Figure 1 shows that, under approximately identical conditions, the various stages lose weight at different rates. The first instar, which is the smallest larval instar and with relatively the greatest amount of surface, loses weight most rapidly. The other stages follow in the order of development and metamorphosis, with the exception of the late prepupa, probably because a rapid loss of weight normally occurs in this stage. The graphs in this figure represent averages taken from records of 35 or more individuals of each stage. The average rate of desiccation gradually decreases as the water content of the beetles decreases. This is not very pronounced in the late prepupal stage, probably also because of the rapid loss of weight characteristic of this stage.

Tables I and II show the maximum fatal limits of desiccation, and the percentage of survivors above this limit, for each stage of development and metamorphosis. The larval stages are the most resistant; the first instar survives a desiccation to 50 per cent of its

original weight; the second instar, to 45 per cent; and the third instar, to 50 per cent. Not only is the second instar able to withstand a greater loss of weight, but the percentage of survivors at points above this fatal limit are greater than in the other larval stages. One

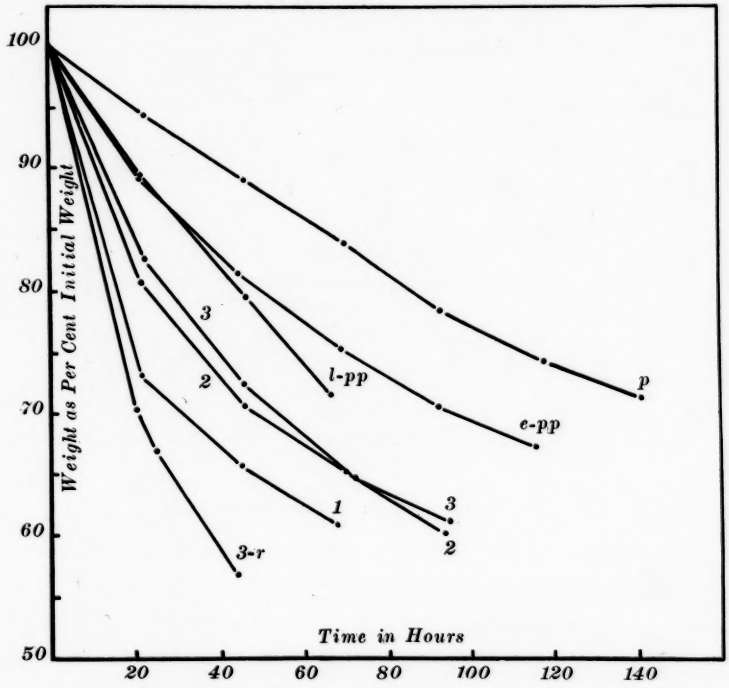

FIG. 1.—Rate of weight loss during desiccation of various stages: *1*, first-instar larva; *2*, second-instar larva; *3*, third-instar larva; *3-r*, third-instar larva, rapid desiccation; *e-pp*, early prepupa; *l-pp*, late prepupa; *p*, early pupa.

hundred per cent of the second-instar larvae survived a weight loss to 56 per cent of their original weight. In the first instar, 71.4 per cent survived the same amount of desiccation; and in the third instar, only 66.6 per cent survived.

Rapid desiccation is more harmful than slow desiccation. In the rapid desiccation of the third instar, the maximum fatal limit was

TABLE I

DESICCATION AND SURVIVAL OF THE LARVA OF THE JAPANESE BEETLE

Extent of Desiccation (Weight, Percentage of Original Weight)	Number of Larvae	Percentage of Survivors
	FIRST-INSTAR LARVA DESICCATED	
Less than 50	6	0.0
50–52	15	20.0
52–54	21	38.0
54–56	18	44.4
56–58	21	71.4
	SECOND-INSTAR LARVA DESICCATED	
45–48	15	13.3
48–50	10	40.0
50–52	20	35.0
52–54	21	66.6
54–56	23	91.3
56–58	25	100.0
	THIRD-INSTAR LARVA DESICCATED	
Less than 50	10	0.0
50–52	24	8.3
52–54	25	20.0
54–56	24	50.0
56–57	12	66.6
57–65	15	73.3
65–70	13	100.0
	THIRD-INSTAR LARVA DESICCATED (RAPID DESICCATION)	
Less than 50	13	0.0
50–52	6	0.0
52–54	17	0.0
54–56	21	14.2
56–58	24	29.1
58–59	11	45.4

54 per cent of the original weight; and only 29.1 per cent survived a weight loss to 56 per cent of their original weight.

As the individual metamorphoses, it becomes less resistant to desiccation; the fatal limits are 56 per cent of original weight in the

TABLE II

DESICCATION AND SURVIVAL OF PREPUPAE AND PUPAE OF THE JAPANESE BEETLE

Extent of Desiccation (Weight, Percentage of Original Weight)	Number of Beetles	Percentage To Form Pupae	Percentage To Form Normal Adults
	EARLY PREPUPA DESICCATED		
54–56	9	0.0	0.0
56–58	22	13.6	9.0
58–60	20	25.0	15.0
60–62	20	40.0	40.0
62–64	20	75.0	60.0
64–66	20	70.0	50.0
	LATE PREPUPA DESICCATED		
64–67	6	0.0	0.0
66–69	6	16.6	0.0
69–71	8	25.0	0.0
71–73	8	62.5	25.0
73–75	8	87.5	50.0
	EARLY PUPA DESICCATED		
64–67	21	.....	0.0
67–69	20	.....	0.0
69–71	20	.....	5.0
71–73	20	.....	15.0
73–75	20	.....	20.0
75–77	20	.....	25.0
77–79	20	.....	70.0
79–81	20	.....	60.0

early prepupal stage, 66 per cent in the late prepupal stage, and 69 per cent in the pupal stage. It is interesting to correlate the slower rate of drying of the pupal stage (Fig. 1) with its higher fatal limit of desiccation (Table II). It appears that, although the pupae are

not able to survive a loss of more than 31 per cent of their weight, they are still able to withstand exposure to drying environmental conditions as long as, or longer than, the other stages, because of the slow rate of water loss characteristic of this stage.

Approximately all of the weight loss in the Japanese beetle is due to the evaporation of water. The average water content of 35 third-instar larvae was found to be 81.0 per cent. Using this figure as normal, the amount of water in partially dehydrated larvae was calculated by assuming that all of the weight lost was due to the loss of water. Thirty-three such partially dehydrated larvae were then completely desiccated, and the observed water content compared with that calculated for the same individuals. The average deviation was 0.4 per cent. Hence, there is evidently no conservation of metabolic water in the Japanese beetle larva.

The water content of the various stages of metamorphosis varied as follows: third-instar larva, 81.0 per cent; early prepupa, 77.1 per cent; late prepupa, 75.9 per cent; and early pupa, 76.5 per cent. Using these figures, the water content was calculated for individuals of various stages when they had reached the maximum fatal limits of desiccation. At this point the third-instar larva contained an average of 62.0 per cent water; the early prepupa, 59.1 per cent; the late prepupa, 63.4 per cent; and the early pupa, 65.9 per cent. When degree of desiccation is measured by residual water content, there is less variation than when measured by total weight loss. When measured by weight, as percentage of original weight, the maximum fatal limits of desiccation varied from 45 per cent in the second instar larva to 69 per cent in the early pupa; whereas, when measured by water content, they varied from 59.1 per cent in the early prepupa to 65.9 per cent in the early pupa.

Table III gives a comparison of the weight loss and water loss occurring during normal metamorphosis and during metamorphosis when early prepupae and early pupae were partially desiccated. The figures in the first column are taken from Ludwig (1931). They show a weight loss of 23.6 mg. in male and 30.2 mg. in female beetles during the change from early prepupa to early pupa. During normal emergence of male and female beetles, there is also a weight loss of 66.5 mg. and 76.1 mg., respectively. When the early prepupae were

desiccated (second column of Table III), the average weight loss due to desiccation of the survivors was 81.7 mg. in male and 99.1 mg. in female beetles, while in the same group the weight loss during the

TABLE III

COMPARISON OF METAMORPHIC CHANGES IN DESICCATED AND NORMAL JAPANESE BEETLES

(Data for normal animals, duration of pupal stage excepted, are from Ludwig, 1931)

(Weight expressed in milligrams)

	Normal (Ludwig, 1931)	Prepupae Desiccated	Pupae Desiccated	
			Normal Emergence	Abnormal Emergence
Number of beetles used:				
Males	29	22	25	56
Females	27	15	45	51
Weight of early prepupae:				
Males	205.5±2.7	216.0±2.48	.....	.....
Females	253.7±3.4	272.7±3.71	.....	.....
Weight of early pupae:				
Males	181.9±2.5	165.4±2.24	183.1±2.48	187.6±1.75
Females	223.5±3.9	209.0±3.51	209.8±2.84	225.4±2.25
Weight loss due to desiccation of early prepupae:				
Males	.....	81.7	.....	.....
Females	.....	99.1	.....	.....
Weight loss, early prepupa to early pupa:				
Males	23.6	50.6	.....	.....
Females	30.2	63.7	.....	.....
Weight of adults:				
Males	114.3±1.9	107.0±1.72	111.3±2.05	131.3±1.77
Females	146.3±2.6	140.3±2.88	128.1±2.16	158.0±2.04
Weight loss due to desiccation of early pupae:				
Males	.....	.....	37.0	54.2
Females	.....	.....	41.7	63.2
Weight loss on emergence:				
Males	66.5	58.9	34.7	2.0
Females	76.1	70.1	39.5	4.1
Percentage of water in adults	66.6±0.62	66.9±0.44	68.0±0.29	.....
Duration of pupal stage (days)	9.75±0.04	10.5±0.06	11.0±0.06	12.1±0.05

change from early prepupa to early pupa was only 50.6 mg. and 63.7 mg., respectively. Thus there appears to be a partial recovery of water, amounting to more than 30 mg., during this stage of metamorphosis. Normally, this stage is characterized by a slight loss of

water. In this series, 58.9 mg. and 70.1 mg. were lost during the emergence of male and female beetles, respectively, the adults possessing the normal water content of 66.9 per cent.

When early pupae were desiccated (third column of Table III), the emergence of normal adults was accompanied by a loss of only 34.7 mg. and 39.5 mg. in male and female beetles, respectively. In this series, 37.0 mg. and 41.7 mg. were lost by desiccation. Hence, the total weight loss from early pupa to adult was 71.7 mg. in the males and 81.2 mg. in the females. Practically no recovery of lost water occurred during the pupal stage. In this series, the water content of the adult beetles averaged 68.0 per cent. Therefore, regardless of whether the prepupae or pupae were desiccated, the normal water content of the adult was restored by a loss of less water at the time of emergence. When the larvae were desiccated, complete recovery of lost water was necessary before development could proceed to the next stage.

The fourth column of Table III gives the results obtained when pupae were desiccated too far to permit them to develop into normal adults. The pupae seemed to develop normally, the adults forming within the pupal skin. The color of the head, thorax, and legs changed to the dark green characteristic of normal pupae before emergence. However, the only change which occurred at the time of emergence was the movement of the legs which resulted in freeing them from the pupal skin. All other parts of the body remained within the pupal skin. A similar condition was described by Ludwig (1928) for pupae subjected to extremes of temperature. Table III shows that on the emergence of these abnormal adults practically no water was lost.

Another important effect of desiccation was to increase the duration of the pupal stage (transverse row at the bottom of Table III). At 25° C. the pupal stage in the normal individual requires 9.75± 0.04 days. When early prepupae were desiccated, it required 10.5± 0.06 days. In this series there had been a partial recovery before pupation. When early pupae were desiccated, the pupal stage required 11.0±0.06 days in those cases where normal adults were produced and 12.1±0.05 days when abnormal adults resulted. In the last group there had also been a much greater water loss.

The time required for recovery from desiccation in third-instar larvae was correlated with original weight, total loss of water, and rate of desiccation. These observations are summarized in Table IV. They indicate that the greater the degree of desiccation, the

TABLE IV

EFFECT OF VARIOUS FACTORS ON THE TIME FOR RECOVERY FROM DESICCATION OF THE THIRD-INSTAR LARVA

DEGREE OF DESICCATION

Extent of Desiccation (Weight, Percentage of Original Weight)	Number of Larvae	Average Time for Recovery at 25° C. (Days)	Min. Time (Days)	Max. Time (Days)
50–55	14	22.5	14	30
55–60	21	18.7	8	32
60–65	12	14.5	6	27
65–70	14	8.7	3	19

WEIGHT OF LARVAE

Weight of Larvae (Mg.)	Number of Larvae	Average Time for Recovery at 25° C. (Days)	Min. Time (Days)	Max. Time (Days)
Less than 200	20	15.2	3	29
More than 200	41	17.1	3	32

TIME OF DESICCATION

Time for Desiccation (Hours)	Number of Larvae	Average Time for Recovery at 25° C. (Days)	Min. Time (Days)	Max. Time (Days)
Less than 72	33	14.0	3	27
72–100	16	15.4	6	28
100–150	15	21.0	11	30
More than 150	4	25.6	21	32

longer it will require for complete recovery. Size of the larva has no influence on recovery, while those larvae which became desiccated in a shorter time also required less time for recovery. This is probably due to the fact that, in general, those larvae which required a longer time for desiccation had also lost more water. The principal factor

concerned with the time of recovery seems to be the extent of desiccation.

DISCUSSION

One of the most obvious features disclosed by these studies was the tremendous individual variation in the rate of desiccation under approximately the same environmental conditions. Similar variations were reported by Gunn (1933) for the cockroach, *Blatta orientalis*. He found that in dry air at 30° C., one animal lost 5.5 per cent of its weight per day and lived for 8 days, while another lost 32.2 per cent and died in 1 day. This range corresponds to that reported in the present paper. Gunn states that the most careful attention to physical conditions hardly reduced the variation at all, and that during the course of a very large number of experiments the real cause of much of the variation was not discovered.

The curves of Fig. 1 show that each stage loses water more rapidly at the beginning of the desiccation period and that the rate of water loss progressively decreases. Buxton (1930), working with the meal worm, *Tenebrio molitor*, and Mellanby (1932*b*) with the bedbug, *Cimex lectularius*, also reported progressive decreases in the rate of water loss. Mellanby believes that it may be due to a decrease of metabolic rate as desiccation and starvation proceed. In the present experiments, it was noted that the activity of the larvae decreased and that they were practically quiescent by the end of the first day. It seems likely that this quiescent condition was associated with a decrease in metabolic rate and that it might result in a decrease in the rate of water loss. Other evidence for this view is the relatively slow rate of desiccation of prepupal and pupal stages. The transformation to the prepupal stage is characterized by a decrease in metabolic rate; and the metabolic rate of the early pupa is very low, being represented by the low part of the U-shaped curve characteristic of metamorphosis (Ludwig, 1931).

The maximum fatal limits of desiccation of the Japanese beetle larvae are very similar to those reported by Breitenbecher (1918) for the potato beetle, *Leptinotarsa decemlineata;* by Hall (1922) and by Buxton (1930) for the meal worm, *Tenebrio molitor;* by Speicher (1931) for the Mediterranean flour moth, *Ephestia kuehniella;* and by Buxton (1932) for the bug *Rhodnius prolixus*. This comparison is

very interesting, since the Japanese beetle lives in moist soil and is normally never exposed to drying environmental conditions. Its ability to withstand slow desiccation undoubtedly assists it in surviving those unfavorable environmental conditions resulting from periods of deficient rainfall. On the other hand, both the meal worm and the Mediterranean flour moth live in dry atmosphere, and their food is low in moisture content. They are adapted to this type of environment by the ability to conserve metabolic water, so that when 50 per cent of the body weight is lost by desiccation the normal ratio of water to dry material is maintained. The Japanese beetle is not able to conserve body water. When it becomes desiccated, the loss of weight is due to a loss of water, the percentage of body water decreasing until death results.

At the present time, no definite information is available regarding the effects of soil moisture on the distribution of Japanese beetle larvae. However, concerning the egg stage, certain observations of Fox (1934) may be significant. He found that the beetle population in heavily infested areas was greatly reduced in 1933 as compared with 1932. He suggests that this reduction may be due to the deficient rainfall during the preceding summer at the time oviposition and hatching were at a maximum.

The change in water content accompanying metamorphosis from larva to adult parallels that reported by Ludwig (1931). The readings are, in most cases, slightly higher; but the average differences are never more than 2 to 3 per cent.

The results indicate that during metamorphosis there is a considerable range in the maximum fatal limits of desiccation, when expressed in terms of weight and as percentage of original weight. However, when expressed in terms of water content, the variations are not as great, ranging from 59.1 per cent body water in the early prepupa to 65.9 per cent in the early pupa. In a form, such as the Japanese beetle, where the loss of weight is due to the loss of water, the extent of desiccation is therefore more accurately expressed in terms of water content than by percentage of original weight lost by desiccation.

It is evident from the experiments that prepupae and pupae contain more water than is necessary for normal metamorphosis. When

early prepupae were desiccated, only a partial recovery occurred before metamorphosis proceeded. Normally, dehydration occurs during this stage; but under the condition of the experiments, an actual absorption of water occurred. When early pupae were desiccated, there was no recovery of lost water; but development continued, although somewhat retarded, until the emergence of the adult. This experiment shows that, even though male pupae may lose an average of 37.0 mg., and female pupae an average of 41.7 mg. of water, they still emerge normally. These values represent about one-half of the water normally lost at the time of emergence. An average loss of more than this amount of water resulted in the inability of the adults to emerge.

The increase in the duration of the pupal stage with decrease in water content of the pupae is in agreement with the observations of many other investigators. Headlee (1917) found that the velocity of development of the eggs and pupae of the grain moth, *Sitotroga cerealella*, and the bean weevil, *Bruchus obtectus*, varied inversely with the relative humidity of the air; whereas, in the larva and in the life-cycle as a whole, it varied with humidity. Hefley (1928) stated that the length of the pupal period of the tachinid *Winthemia quadripustulata* is greatest at a humidity of 7.1 per cent and decreases as the humidity is increased up to 73.4 per cent; and Janisch (1930) showed that the eggs of *Prodenia littoralis* developed most rapidly at 90–95 per cent humidity. On the other hand, Elwyn (1917) concluded that air humidity has no influence on the velocity of development of *Drosophila ampelophila;* and Headlee (1914) concluded, as a result of his experiments on the aphid, *Toxoptera graminum*, that air humidity does not effect the development of those insects which feed on succulent food. However, in the foregoing cases the rates of development were correlated with atmospheric humidity, but the insects were not desiccated, as in the present experiments. Speicher (1931) found that in the Mediterranean flour moth, a dry atmosphere and dry food diminishes size and greatly delays pupation. Speicher's experiments were also not comparable with the present ones, since he found that as long as the larvae and pupae survived they maintained a constant ratio of body water to body weight. On the other hand, the weight lost by the Japanese beetle during desic-

cation actually consisted of a loss of water. In general, it appears that desiccation is always accompanied by a decrease in the rate of development, regardless of whether it results in any change in relative water content.

SUMMARY

1. The rate at which different stages of the Japanese beetle become desiccated, under approximately identical environmental conditions, decreases in the following order: first,- second-, third-instar larvae, late prepupae, early prepupae, and pupae.

2. The maximum fatal limits of desiccation, expressed in weight and as percentage of original weight, varied as follows: second-instar larvae, 45 per cent; first- and third-instar larvae, 50 per cent; early prepupae, 56 per cent; late prepupae, 66 per cent; and pupae, 69 per cent. When expressed in terms of water content, the variations were greatly reduced, as shown by the following figures: third-instar larvae, 62.0 per cent; early prepupae, 59.1 per cent; late prepupae, 63.4 per cent; and pupae, 65.9 per cent.

3. The water content of the adult beetle is approximately the same (66–68 per cent) regardless of whether it had been previously desiccated in the prepupal or the pupal stages. Water content is restored by a loss of less water at the time of emergence. When larvae are desiccated, water content is restored before development can proceed.

4. The prepupal and pupal stages contain more water than is necessary for normal metamorphosis. Pupae can be desiccated until they lose about one-half of the water normally lost at the time of emergence, and still emerge normally.

5. The duration of the pupal stage is increased by desiccation of either the prepupal or pupal stages. This increase is inversely proportional to the water content of the pupae.

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ELECTROKINETIC STUDIES OF MARINE OVA. III. THE EFFECT OF DILUTION OF SEA WATER, AND OF SODIUM AND CALCIUM UPON THE SURFACE POTENTIALS OF ARBACIA EGGS

(One figure)

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IT HAS long been known that the electrokinetic potential of a colloid is affected by various factors in the ionic composition of the medium. Among these, the concentration factor and the valency factor are of great importance; and they have repeatedly been studied by many investigators in the field of colloid chemistry (Abramson, 1934; Freundlich, 1930). The object of the present paper is to examine similar relationships, but in connection with the surface potential of marine ova.

The material used is the eggs of the sea urchin, *Arbacia punctulata*, obtained at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the summer of 1934. The method of measuring the cataphoretic potential here employed is essentially the same as was described by the author in a previous paper (Dan, 1933). The zeta potential was calculated by the classical equation

$$u = \frac{DH\zeta}{4\pi\eta},$$

where u is the migration velocity in centimeters per second; η, the coefficient of viscosity of the medium; D, the dieletric constant of the medium (here this is taken as 80); H, the potential gradient in electrostatic units; and ζ, the zeta potential, also in electrostatic units. However, ζ will be given in millivolts.

The viscosity of various solutions was measured by a viscosimeter of the Hess type and was made comparable to that of sea water. The strength of the electroendosmotic currents in these solutions also varied widely from one case to the other. The corrections for these

differences were calculated from direct observations of the electroendosmotic currents in the solutions by using quartz particles. However, when a series of solutions was employed, each member of which differed in composition by slight gradations, the measurements were not extended to every solution of the series. In these cases, only the correction values for the more important steps were directly obtained, and the values for the intermediate steps were later calculated from a smooth curve which connects the known points.

It will also be mentioned here that, in the experiments which follow, eggs were used from which the jelly had previously been removed. Therefore, when the surface charge is mentioned, it refers to the charge on the surface of the cell and not the charge on the jelly, unless otherwise stated.

DILUTION EXPERIMENTS

In using biological material such as sea-urchin eggs, precautions must be taken to keep the osmotic pressure as nearly normal as possible through the course of the experiment. This fact restricts the range of variation of the ionic strength of the medium a great deal. In the dilution experiment, normal sea water was diluted to 85, 70, and 55 per cent; and the potentials were measured in the respective cases. As is well known, the eggs change their volume in different concentrations. Data for the volume change were taken from a curve in the paper of McCutcheon, Lucké, and Hartline (1931); and the corrections for the electroendosmosis involved in the cataphoretic measurements of large particles (see Dan, 1933, p. 483) were calculated according to their figures.

Fifty-five per cent dilution is not less than half the original concentration; and this may be too insignificant a change in comparison with typical concentration studies, which often cover the range from zero concentration up to 1 molar or more. For this reason, it was suspected from the beginning that there would be no detectable change before and after the dilution within this range. Yet this series was studied for two reasons: First, since these concentrations have been tried in many other physiological studies, it may not be useless to run the parallel experiments in cataphoretic measurements. Second, the author suspected that an interesting find-

ing of Lucké, Hartline, and McCutcheon (1931), that the permeability constant of *Arbacia* eggs for water is different for exosmosis and endosmosis, might have some connection with the change in the electrokinetic potential, inasmuch as recent studies tend to indicate that the elasticity of the cell membrane is negligibly small (E. N. Harvey, 1931; Kamada and Yamamoto, 1931; Cole, 1932; Cole and Michaelis, 1932). The result turned out to be negative, and the potentials in these concentrations are practically identical with each other. However, it will be stressed once more that this does not necessarily mean that the concentration effect does not exist for sea-urchin eggs. It might have been detected, if sufficient range of dilution could be used. The figures are given in Table I. It is also pos-

TABLE I

THE CATAPHORETIC POTENTIALS OF THE EGGS OF *Arbacia punctulata* IN NORMAL AND DILUTED SEA WATER

The figures are given in millivolts with the standard errors

100 per Cent	85 per Cent	70 per Cent	55 per Cent
−30.3±0.47 (1932)	−30.2±0.54	−29.1±0.78	−29.7±0.58
−29.9±0.57 (1933)	.....	.....	.....
−29.7±0.65 (1934)	.....	.....	.....

sible to concentrate the sea water and examine the effect of it on the zeta potential. But, so far, this has not been tried.

SODIUM-CALCIUM COMBINATIONS

For the study of the valency effect, sodium and calcium were used. As was mentioned beforehand, if it is possible to construct two curves, one for NaCl and the other for $CaCl_2$, from zero up to any desired concentrations, then by comparing the two the valency effect can be studied in a simple way. However, since the osmotic pressure of the medium should be kept constant for the present material, the following procedure is the only method available which enables us to investigate the valency effect. This is to take isotonic NaCl* as one extremity and isotonic $CaCl_2$† as the other ex-

* ½ M NaCl rendered alkaline by $NaHCO_3$. Ag-free NaCl by Merck was used.

† ⅓ M $CaCl_2$. The solution of "Baker's analyzed" preparation gives the reaction of pH *ca.* 8.1, owing to $Ca(OH)_2$ present.

tremity of the experimental conditions. For intermediate steps, both solutions were mixed in appropriate ratios. This makes it possible to change the calcium content without upsetting the isotonicity of the resulting mixtures. Then a curve can be drawn relating the potentials and calcium concentrations. But as is clear, the curve thus obtained is not comparable with the above-mentioned NaCl- or $CaCl_2$-curve, respectively. This curve corresponds to one which connects two points, one on a NaCl-curve at 1/2 M to the other on a

TABLE II

THE ZETA POTENTIALS IN VARIOUS SODIUM-CALCIUM MIXTURES AND UNDER OTHER RELATED CONDITIONS

Figures are given in millivolts with standard errors

Solutions	Potential Difference	No. of Cells Measured
Sodium-calcium series:		
1/3 M $CaCl_2$	−13.7±0.57	62
1/6 M $CaCl_2$ in NaCl	−21.6±0.73	59
1/10 M $CaCl_2$ in NaCl	−25.0±0.59	54
1/25 M $CaCl_2$ in NaCl	−27.5±0.67	55
1/175 M $CaCl_2$ in NaCl	−30.0±0.61	59
1/250 M $CaCl_2$ in NaCl	−29.5±0.74	78
1/1,000 M $CaCl_2$ in NaCl	−25.5±0.53	40
1/2 M NaCl	−20.3±0.55	50
First washed in NaCl (potential difference measured in sea water)	−19.7±0.54	64
First washed in urea (potential difference measured in sea water)	−20.5±0.44	69
Sea water	−29.7±0.65	71
Calcium-free sea water	−31.0±0.64	79

$CaCl_2$-curve at 1/3 M. Moreover, as the curve is followed from the NaCl end to the $CaCl_2$ end, every three ions of sodium are replaced by two ions of calcium. In spite of all these drawbacks, it should be possible to detect qualitatively the existence of the valency effect, if it occurs. The result shows clearly that this is the case.

The data are summarized in Table II, and a curve constructed from them is shown in Figure 1. (The reader's attention will more conveniently be called to this figure in a later discussion.) The ordinates in the figure represent the zeta potentials in millivolts,

and the abscissae represent the ratios of the mixtures of isotonic NaCl and isotonic $CaCl_2$, the ratios being shown as the final calcium concentrations in the resulting mixtures. The values shown in a special section are those obtained in sea water. ●'s are values observed for living cells in sodium-calcium mixtures, ▲'s are those for cytolyzed cells. ▼ is for the cells treated with urea before transferring them to sea water, and ■ is for those treated with Herbst calcium-free sea water.

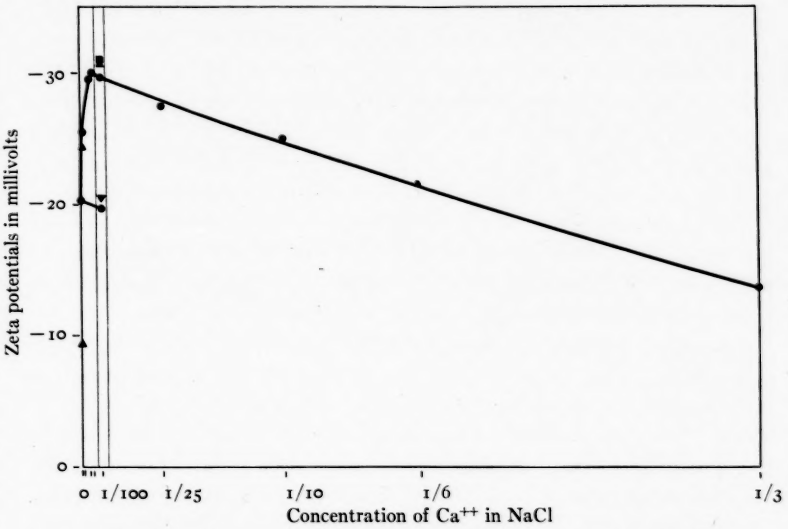

FIG. 1.—The effect of sodium and calcium ions on the zeta potentials of *Arbacia* eggs

As a starting-point, it will be convenient to choose the value in normal sea water. Strictly speaking, this point does not belong to the present series because of the Mg, SO_4 and other ions found in sea water besides Na, Ca and Cl. However, this figure is given because this is a convenient value for the purpose of comparison, since in some cases eggs were returned to sea water after having been in various other solutions. Moreover, this is the most reliable figure, as it has been repeatedly confirmed in the past three years. The value of the zeta potential for normal sea water will temporarily be placed on the curve in a special section at the level of 1/100 M according to

its calcium content, neglecting Mg and SO_4, etc. (Wheeler, 1910). At any rate, this point serves simply as a reference point.

In the beginning, the higher concentrations of calcium will be examined. As is clear from the graph, when calcium is made more and more concentrated, the absolute magnitude of the negative potential on the surface of the egg cell decreases. When finally 1/3 M $CaCl_2$ is reached, the zeta potential, which was about -30.0 mv. at the start, now becomes -13.7 mv. This decrease in the absolute magnitude of the negative potential is obviously the ionic effect due to calcium ions. However, stronger support for this explanation can be found in the following fact: If a measurement of the potential in one solution is first made and then the same eggs are transferred to another different solution, the potential can be shifted either to a higher or a lower level according to the concentration of calcium in the second solution. Values for 1/6 M and 1/25 M on the curve were thus obtained. Eggs used in both cases were derived from the same female. They were put in 1/6 M first, and after the measurement they were then washed with 1/25 M calcium. The interval between two sets of experiments was about 10 minutes, which was the minimum time required to secure a complete change of solution (four washings). This demonstrates clearly that on this side of the curve the magnitude of the potential is a function of the composition of the outer medium, and not of anything inherent in the cell membrane.

Contrary to this, the situation is very much different on the left-hand side of the curve. At 1/175 M and 1/250 M, the curve remains practically level. But when it approaches 1/1,000 M, the absolute magnitude begins to descend; and in 1/2 M NaCl it drops to -20.3 mv. If the valency effect is the only factor at work, the absolute magnitude of the potential ought to be the highest in 1/2 M NaCl, for there are no divalent cations in this solution. This statement is true either with inanimate particles, such as quartz, or with living cells when the surface is believed to be non-reactive with the medium (Winslow, Falk, and Caulfield, 1923; Mudd, Mudd, and Keltch, 1929). Now, if the eggs, the potential of which has been once lowered in NaCl, are returned to normal sea water, their potential does not return to the original level but stays permanently low. This makes a strong contrast to the aforementioned change in potentials

in the region of higher calcium concentration, which is characterized by its complete reversibility. This piece of evidence decisively shows that the drop in potential in NaCl is not a simple ionic proposition but must be something more inherent in the nature of the cell membrane. Or, in other words, it suggests that something irreversible has happened or else that something must have been added to or removed from the surface by washing the cells in NaCl. More detailed discussion will be given later; but it may be mentioned here that the author inclines to believe, for the present, that this is due to a permanent loss of the outermost layer.

CYTOLYZED EGGS

In the second paper of this series (Dan, 1934), the potentials of living and dead *Asterias* eggs were compared; and it was shown that

TABLE III

THE ZETA POTENTIALS ON LIVING AND CYTOLYZED *Arbacia* EGGS IN SODIUM-CALCIUM MIXTURES AND IN SEA WATER

Potential differences are given in millivolts with standard errors

	Normal Sea Water	NaCl Solution with 1/1,000 M Calcium-Ions	1/2 M NaCl
Living	−29.9±0.57	−25.5±0.53	−20.3±0.55
Cytolyzed	−30.7±0.68	−24.3±0.51	− 9.4±0.45

no change could be found between the two. In the same year, a similar experiment was tried with *Arbacia* eggs, and here also the potentials on heat-killed and living *Arbacia* eggs were found to be identical. Therefore, this seems to be a general phenomenon among marine ova. During the last summer more measurements of the same sort were attempted, and the potentials in a mixture of NaCl and 1/1,000 M $CaCl_2$ and in 1/2 M NaCl alone were studied. The figures for the foregoing three cases are tabulated in Table III.

Concerning the method for obtaining cytolyzed eggs, it will be mentioned that in the case of normal sea water, eggs were subjected to 40° C. for 5 minutes. In the other two cases, because of the extremely low calcium content, the cell membranes became so weak that a slight stirring of the egg suspension caused many eggs to

cytolyze. When the eggs do cytolyze, however, the mode of cytolysis in the last two solutions is totally different. The former contains calcium ions up to 1/1,000 M. Therefore, when eggs cytolyze in this solution, they form a typical "surface precipitation membrane" in Heilbrunn's sense (Heilbrunn, 1927); while in 1/2 M NaCl, protoplasm streams out without forming any membrane. In NaCl this condition of the eggs is called "cytolysis." Therefore, strictly speaking, it is the potential of protoplasmic granules in NaCl which is measured. As will be noticed immediately, Table III shows that, as long as there are calcium ions, potentials on living and cytolyzed eggs behave correlatively, while only in the absence of calcium ions cytolyzed egg cells (or protoplasmic granules) acquire an entirely different potential from that of the intact cell surface.

DISCUSSION

The measurements of cataphoretic potentials in sodium-calcium mixtures show two main facts. The first is that when calcium ions are contained in a medium beyond a certain critical value, the zeta potentials of *Arbacia* eggs behave like potentials on inanimate colloids; i.e., the absolute magnitude of the negative potential decreases when the concentration of divalent cations increases. And, also, this shift in potential is completely reversible. It can be changed at will in either direction by regulating the calcium concentration of the medium. The second fact is that below the critical value of calcium ions, the absolute magnitude of the negative potential on the egg cells again drops, but this drop is very sudden and irreversible. A close examination of these results led me to conclude that this irreversible change in potential in NaCl is caused by a permanent loss of some coating layer from the cell surface.

There are innumerable facts indicating that the cell surface reacts with calcium ions. It is quite a universal fact that the cell membrane is weakened a great deal if calcium is removed from the medium. The well-known discovery of Herbst (1900) that blastomeres of *Echinus* fall apart in calcium-free sea water indicates that the cell surface reacts with calcium, thus giving rise to a cement substance which is probably a calcium gel of some sort. Galtsoff's study on the aggregate formation from dissociated cells of *Microciona* (1925)

shows that a similar mechanism is involved in this phenomenon. Gray investigated the dissolution of the cell matrix in *Mytilus* gills (1926). He emphasized the predominant rôle played by magnesium in securing the normal condition of the tissue. But even in his case, he mentioned that calcium ions are necessary to make the complete stability of the tissue possible for more than 48 hours. Besides these, the fertilization reaction which involves a series of changes in the cortex is known to be impossible in the absence of calcium. It is also known that the hyaline plasma layer fails to appear if fertilized eggs are transferred to a calcium-free medium immediately after fertilization. Moreover, similar facts are frequently met with among plant cells. Hansteen Cranner's classical papers elucidated the problem more successfully than in any other cases (Hansteen, 1910; Hansteen Cranner, 1914); and other works with their reviews can be found in his papers. Benecke's work (1898) also led to a conclusion of the same sort.

A slightly different aspect of a similar phenomenon is approached by Heilbrunn in his study of the surface precipitation reaction. This is also a widely occurring phenomenon among different types of protoplasm (see Heilbrunn, 1928, chap. 13). More recently, Weber (1932) drew attention to an analogous behavior in *Spirogyra*. These facts, enumerated above, may not necessarily belong to a single category. Most likely they may represent diverse reactions occurring on the surface. But it suffices if they can illustrate how frequently calcium ions react with protoplasm at the boundary surface.

With these facts in mind, it is not surprising to think that there is a coating of a calcium gel on the surface of the normal *Arbacia* eggs, even though its existence cannot be detected by microscopical techniques at present. If this is admitted, it is not hard to imagine that this layer will be dissolved in case calcium is removed from the medium. Peptization of this hypothetical calcium gel in calcium-free media is again paralleled by the dissolution of calcium mucinate (Gray, 1931) and calcium caseinate (Robertson and Miyake, 1916) among inanimate substances. And of great interest is the fact that the eggs of sea urchins have two kinds of substances which actually behave in the same manner. The one is the surrounding jelly. As everybody knows, NaCl dissolves the jelly. The other is the ecto-

plasmic layer of the fertilized cells. As far as the writer is aware, E. B. Harvey (1934) was the first who noticed it. Quoting her paper (p. 232), she states:

> If the eggs after removal from the centrifuge are left in the same solution (i.e., minus Ca) or transferred to calcium-free sea water, the perivitelline space remains perfectly clear and there is no ectoplasmic layer on the surface of the eggs. If the eggs, on removal from the centrifuge, are put in normal sea water, the perivitelline space becomes filled with many small refringent spherical or oval bodies (Fig. 7), the precipitation product of the ectoplasmic material in the presence of calcium. On return to calcium-free sea water, these are again dissolved and can be precipitated again in the presence of calcium. The ectoplasmic material, after being centrifuged off as a ring, may also be dissolved in calcium-free sea water and be precipitated again as scattered spherules when returned to sea water.

My friend, Mr. T. Yanagita, quite independently noticed this reversible precipitation and dissolution of the hyaline plasma layer in the eggs of *Strongylocentrotus pulcherrimus*, and he has been comparing the potencies of various salts to cause a reprecipitation of the dissolved ectoplasmic material. He is so generous as to permit me to quote a part of his unpublished data. It is sufficient for me to mention here that Mr. Yanagita found that magnesium can also precipitate the ectoplasmic material but that the minimum concentration required for magnesium to cause the precipitation is about 60 times greater than that required for calcium.

Let us view the whole thing from this standpoint. From the foregoing illustrations it is safe to say that the cell surface of sea urchin eggs reacts with calcium. At the critical amount of calcium, the surface is just completely covered by the calcium gel. On increasing the calcium content beyond this value, the nature of the cell surface cannot be changed any further. The surface is saturated with calcium, so to speak. The thickness of the coating layer might become greater, but still its nature will remain unchanged as far as the electrokinetic properties are concerned. Then, for higher concentrations of calcium, the cell surface of *Arbacia* can be looked upon as an inert surface; and it would be expected that it ought to behave like any inert colloidal particle, that is, the zeta potential ought to be entirely dependent upon the composition of the medium. This is demonstrated clearly in our result.

Now for dilute concentrations, this gel may begin to be dissolved. At 1/1,000 M calcium, the curve is halfway down to the NaCl level. This may indicate that the surface is only partially covered by the gel, leaving the "naked" surface exposed to some extent. Finally in 1/2 M NaCl, this "naked" or underlying surface alone is being dealt with. Pushing one step forward, if this be true, NaCl may not be the only reagent which can dissolve this outer layer. Any solution devoid of calcium would serve for the same purpose. Isotonic urea was chosen for the test. In this case, the potential was not measured in urea because of its being a non-electrolyte. But the cataphoretic measurement was made after returning the eggs to sea water. As is shown on the graph, it turned out to be -20.5 mv., thus coming very close to the value of eggs treated with NaCl. The effect of sugar seems to be in the same direction, even though no quantitative study was made (see "Electrokinetic Studies of Marine Ova. IV" [this journal, p. 58]).

The nature of this outer layer of calcium gel seems to be very much the same as that of the surface precipitation membrane of Heilbrunn. As a matter of fact, it is not possible to differentiate between the two as far as the cataphoretic behavior is concerned. Probably the reason why the death of the cells does not modify the zeta potential in either *Arbacia* or *Asterias* eggs will find its explanation in the following way. At the moment the cell membrane loses its semi-permeability by death, the surface precipitation reaction is propagated throughout the cytoplasm, thus enveloping the dead cytoplasmic mass with the surface precipitation membrane, which is presumably a kind of calcium gel. Then it follows that we are dealing with the same calcium compound either in intact cells or in dead cells. Heilbrunn reported in his recent work (1934) that the inhibiting effect of anesthetics on the surface precipitation reaction can be observed in a very low concentration of calcium. He writes that this antagonism cannot be observed in normal sea water, and he ascribed it to an overwhelming abundance of calcium in sea water. This may indicate, even though indirectly, that in the low concentration he used the surface precipitation reaction is barely happening. Therefore 1/1,000 M was chosen, and the cataphoretic potentials of both normal and cytolyzed eggs were measured in this concentration. The

result is very interesting. The potentials on both normal and cytolyzed eggs decreased in their absolute magnitude, and they did it to the same extent (see Table III). This means that when the surface precipitation membrane is only partially formed, owing to a low calcium content, the outer gel layer is also partially destroyed, and the effect on both seems to be to the same degree. In other words, the decrease in the absolute magnitude of the negative potential indicates that in these cases the underlying surface has begun to show its effect (see Dummett and Bowden, 1933; Moldowskaya, 1933), and the fact that both turned out to be about -25.0 mv. suggests that the ratio of the area of the surface covered by the gel and that left uncovered must be about equal in both cases. Coming this far, it can easily be expected that if there is an experimental condition in which the difference between the cell surface and the internal protoplasmic granules becomes evident, it must be in some medium where this gel can be removed completely, for if a film of the gel remains, it may camouflage the underlying surface. This expectation proved to be true. As is shown in Table III and in Figure 1, in NaCl—and only in this solution—the living cell surface and the protoplasmic granules show widely different potentials.

The preceding experiments naturally led me to try the effect of Herbst calcium-free sea water. Somewhat contrary to my expectation, the value turned out to be practically the same as the normal one. Probably the explanation may be as follows: This calcium-free sea water contains a fairly high amount of magnesium, a much higher concentration than the critical value of magnesium for precipitating the ectoplasmic material in Mr. Yanagita's experiment. Thinking from the fact that the peptization of calcium mucinate is also retarded in the presence of divalent cations, it is very plausible to think that the calcium gel was protected from dissolution by magnesium ions contained in Herbst calcium-free sea water, or at least it must have required a much longer time for the gel to be dispersed than under the ordinary condition. When this series of experiments was being conducted, the time factor was not taken into consideration. Usually, eggs were left in solutions from 10 to 60 minutes before potentials were measured. It is hoped that an opportunity will be found to investigate this point more fully in the future.

Summarizing, in higher concentrations of calcium ions, either from the complete reversibility of changes in potential or from the general shape of the potential curve, it is clear that we are dealing with the ionic effect on an inert surface. On the side of greater dilutions, we notice the striking fact that potentials of *Arbacia* eggs tend to group themselves into three classes under widely diverse conditions: the first is the level of -30 mv. (intact cells, heat-killed cells, and cells in calcium-free sea water belong to this group); the second is the level of -20 mv. (eggs washed in NaCl and measured in NaCl, the same measured in sea water, and cells treated with urea and measured in sea water); and the third is the level of -10 mv. (cytolyzed cells in NaCl). This fact indicates that here we are dealing with three distinctly different surfaces, namely, the outermost gel layer, the "true" cell membrane, and the surface of the internal granules.

I acknowledge my indebtedness to Dr. L. V. Heilbrunn, whose kind suggestions and vivid discussions I found most inspiring.

SUMMARY

1. The concentration effect and the valency effect of the medium on the zeta potentials of *Arbacia* eggs were investigated.

2. No effect of dilution could be detected within the range of dilution employed in the present study (between 100 per cent and 55 per cent sea water).

3. The study of the effect of isotonic mixtures of NaCl and $CaCl_2$ in various ratios brought out the following facts:

a) In higher concentrations of calcium, the surface of *Arbacia* eggs behaves as an inert surface, that is, the absolute magnitude of the negative potential decreases when the concentration of divalent cations increases; and this change is reversible.

b) In an isotonic solution of NaCl, the absolute magnitude of the negative potential again drops, but it does so irreversibly.

c) In very low concentrations of calcium, the potential shows an intermediate value between those in sea water and in NaCl.

4. The comparison of the potentials on intact and cytolyzed cells shows that they have practically the same potentials and that, when they change, they do so *pari passu*, as long as calcium is present in

the medium. Only in the absence of calcium do the two potentials differ widely from each other.

5. The facts described in 3 and 4 find a simple explanation by assuming the existence of a calcium compound on the cell surface which is dispersed in the absence of calcium ions.

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ELECTROKINETIC STUDIES OF MARINE OVA. IV. THE SURFACE POTENTIALS OF CENTRIFUGED FRAGMENTS OF ARBACIA EGGS

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WE ARE familiar with numerous experiments in which centrifugal force was applied to cells of various types. If we restrict ourselves to egg cells for the present, we find that practically all the classical papers seem to agree in two main points: first, the internal granules can easily be shifted in position; second, the cortical layer, on the other hand, is not so easily affected (at least by hand centrifuges).

More recently, however, owing to technical improvements, it has become relatively easy to employ much stronger forces. As a result of this advancement, evidences now seem to be accumulating that the cortical material also suffers a shifting if much stronger forces are applied on it.

Lindahl (1932), in his dark-field examinations of centrifuged eggs of *Arbacia pustulosa*, recognized three layers on the surface of unfertilized eggs. These are the outer shining layer, the middle dark layer, and the innermost shining layer. This innermost layer is the lipoid layer, according to Runnström (1923). In his extensive studies (1928), Runnström claimed that *Arbacia* was exceptional in lacking this shining layer. However, Lindahl, after confirming the former's description in uncentrifuged eggs, discovered that this layer is detectable after centrifuging. As is clear from Lindahl's figures, the two inner layers thin out toward the centripetal pole and thicken toward the opposite end. At any rate, it is beyond doubt that the cortical material changes its normal distribution after centrifuging.

A more spectacular case of a similar kind was reported by E. B. Harvey (1934). She found that the ectoplasmic layer of fertilized sea-urchin eggs can be thrown off the eggs completely in a ring (*Parechinus microtuberculatus*). This fact indicates that the cortical

layer is composed of a heavy material which usually maintains its integrity by its high viscosity, but can eventually be thrown down if a strong enough force is applied.

A more important phase of this line of study, however, may be the fact that the membrane-forming capacity of centrifuged eggs seems to be closely correlated with the distribution of their cortical material.

Taylor first reported (1931) that the centrifuged eggs of *Urechis caupo*, on insemination, form more or less triangular fertilization membranes. This is due to the fact that the membrane elevation is very slight at the centripetal end, while it is more conspicuous on the opposite side. Thus the membrane broadens toward the heavy pole. A series of beautiful photographs of Costello's experiments (1935) on starfish eggs speaks for this most clearly and most emphatically. In this case, not only the perivitelline space becomes extremely narrow on the centripetal end but the membrane is missing entirely at this pole. Therefore, in lighter halves, after several cycles of cleavage, blastomeres escape through the opening in the membrane, and they are scattered in the medium.

As to the sea-urchin eggs, Harvey states:

> If broken apart (in tubes at 11,000 times gravity) before the elevation of the fertilization membrane, this membrane may subsequently form over each part, but is thinner (ruptures more easily) over the centripetal pole of the part; it is also thinner over the oil cap of the elongate whole egg [Harvey, 1933*a*, p. 389].

Now, with these facts in mind, a series of experiments was undertaken to see whether or not a change in the electrokinetic potential of the eggs of *Arbacia* accompanies the fragmentation by centrifuging.

MATERIAL AND METHOD

The experiments, the results of which are to be presented in this paper, were performed during the summers of 1933 and 1934 at the Marine Biological Laboratory, Woods Hole, Massachusetts. The eggs of *Arbacia punctulata* were used as material. They were obtained by making ripe females shed by removing the oral parts. The fragmentation by centrifugal force is effected according to Harvey's method, that is, by stratifying the egg suspension over isotonic sucrose solution in centrifuge tubes. After the separation of lighter and

heavier fragments, they were washed several times in sea water. The cataphoretic potential was measured by the method previously described (Dan, 1933). A correction for the electroendosmotic flow, which was also discussed before, eliminates automatically a possible influence of the size difference between lighter and heavier halves on the apparent speed of cataphoretic migration.

When the separation is completed, the jelly is usually thrown off entirely. Therefore, no special treatment was employed for this purpose. However, in order to secure the complete removal of the jelly, the egg suspension was sucked in and out of a pipette during the washing process. The complete removal of the jelly can also be checked by the careful examination of the speed of fall of these fragments at the time of the potential measurements, for eggs with the jelly fall considerably more slowly than those without it. Thus it will be understood that the potentials given below refer to those of eggs or egg fragments with the jelly removed.

RESULTS

In the summer of 1933, egg suspensions (in sea water) were stratified over isotonic sugar solutions in order to get separation into fragments. The potentials on the fragments thus obtained are given in the first row of Table I. As will be seen, the potential on the centrifuged non-fragmented eggs was -26.0 mv.; on the heavy halves, -25.6 mv.; and on the light halves, -21.2 mv. At that time, the author could not give any plausible explanation for these results. However, toward the end of the season, it began to be suspected that the use of isotonic sugar solution itself seemed to modify the potentials of the eggs; and, moreover, various indirect evidences suggested that this was apparently due to the absence of calcium in the sugar solution. Therefore, in the following summer, a similar experiment was repeated, but the isotonic sugar solution was replaced with an isotonic mixture of sugar and $CaCl_2$. The calcium concentration was so adjusted as to be about the same as that occurring in the natural sea water. This causes a prolongation of the time of centrifuging necessary for fragmentation. The potential measurements also showed a slightly different result. The figures are given in the second row of the table. Here the potential on the centrifuged non-frag-

mented eggs came out as −30.8 mv., a more negative potential. As was shown in previous papers, this value is identical with the potential on the normal eggs if the jelly is removed from them. The heavier halves also acquired a slightly more negative potential, and the value for the lighter halves remained practically unchanged.

The writer thinks that in the light of the conception arrived at in the previous paper of this series ("Electrokinetic Studies of Marine Ova. III" [this journal, p. 43]), we are now in a position to give an explanation for the foregoing results. Summarizing the conclusion reached at the end of the previous paper, it is roughly as follows: (1) The surface of the egg cell (jelly removed) is covered by a coating

TABLE I

CATAPHORETIC POTENTIALS OF EGG FRAGMENTS

Figures are given in millivolts with standard errors

	Centrifuged Non-fragmented Eggs	Heavy Fragment	Light Fragment
Experiment performed in 1933 (isotonic sugar was employed)	−26.0	−25.6±0.84	−21.2±0.64
Experiment performed in 1934 (isotonic mixture of sugar and $CaCl_2$ was used)	−30.8±0.54	−27.6±0.35	−20.9±0.69

of a calcium compound. This layer is characterized by a potential of −30 mv. (2) This layer is dispersed in media lacking in calcium. Then an underlying surface becomes exposed. This new layer is characterized by a potential of about −20 mv. (3) When the calcium concentration is extremely low, the potential falls to somewhere between −30 and −20 mv., owing presumably to a partial dispersion of the calcium compound described above.

Now, it is extremely interesting to find that all six values given in the table fall in one of the foregoing three classes. Beginning with the case of centrifuged non-fragmented eggs, when calcium ions are involved uniformly in two stratifying solutions, the potentials on these eggs coincide with the value of the normal uncentrifuged eggs (−30 mv.). But if the addition of calcium to the sugar solution is omitted, the potential of the centrifuged non-fragmented eggs drops

to −25 mv. This potential happened to be the same as was obtained in a mixture of NaCl and 1/1,000 M $CaCl_2$ in the previous paper. Now, when the egg suspension in sea water is stratified over the isotonic sugar solution and centrifuged, a minute amount of calcium from the sea water must, no doubt, have been mixed with the sugar. There are several reasons to believe so.[1] And if this is really the case, we have to expect that this group should acquire a potential characteristic of those eggs which were treated with a medium containing a trace of calcium.

On the contrary, the potentials on the lighter fragments stayed at the level of −20 mv. in both sets of experiments. This fact shows that on the surface of the light fragment, the calcium gel is absent. If so, it naturally follows that the absence or the presence of calcium ions cannot cause any change in potentials, as there is no calcium gel to be affected.

Assuming that the foregoing explanation is correct, this result gives us some insight to the change occurring on the surface at the time of fragmentation. That is: as long as the cells remain intact, the calcium layer seems to cover the entire surface of the cell even after a long centrifuging, for if the layer is partially thrown down, leaving the underlying surface exposed, the absolute magnitude of the resulting potential ought to be less than 30. When, however, the separation has taken place, the lighter halves acquire a potential of −20 mv. This means that on the surface of the lighter halves, the calcium gel is missing. Then the question arises as to where the calcium gel has gone. The present results suggest that the calcium

[1] There are three reasons: (1) When the stratification was made, it was my usual practice to fill the centrifuge tube first with the suspension; then the sugar solution was introduced to the bottom of the tube by a long pipette. Therefore, when the sugar was poured out of the pipette, it pushed the lighter suspension up from below. As a result, it is evident that a small quantity of sea water must have been adhering to the surface of the tube. (2) After the stratification was made, the boundary of the upper sea water and the lower isotonic sugar was intentionally stirred in order to get a gradual transition between the two solutions. This was done because the less steep the gradient of specific gravity change, the more efficient it was to cause the separation of two halves. (3) When the eggs were fragmented, the heavier halves sank to the bottom of the tube through the sugar solution. Therefore, when a great number of the eggs were fragmented at the same time, an appreciable amount of calcium must have been carried with them into the sugar solution.

layer is thrown down to the centrifugal pole of the heavy halves. There are two reasons to support this view:

1. As can be learned from Table I, in the experiments performed in 1934 the heavier halves have a potential of -27 mv. If the calcium layer remains and covers the whole surface area of the heavy halves, the potential must be -30 mv.; while, if this layer is completely cast off, the potential must be -20 mv. The fact that this potential lies between -30 and -20 may indicate that on the surface of the heavier halves both kinds of surfaces are being exposed. If we follow this simplified picture a step farther, it means that one third of the surface is made of the underlying layer; and two thirds, of the calcium layer according to the following simple calculation:

$$21 \times X + 31 \times (1 - X) = 27 \qquad \therefore X = 0.3 .$$

2. Occasionally, eggs were found which broke up into three parts. They are the light fragment which is almost exactly like the ordinary light halves; the middle one, which takes up the most part of the ordinary heavier halves; and the heaviest fragment, which is very small and closely packed with the pigment granules. Harvey (1933*b*) noticed that the sizes of the fragments vary when the centrifugal force employed is changed. And in some of her experiments, she did get "triple eggs." However, the size relationship seems to be very much different in her experiment and in mine, and I obtained triple fragmentation only very seldom. Because of the scarcity of cases, no quantitative data could be taken. But it is beyond any doubt that the middle fragment carries the same potential as the light ones and the tiny heaviest part moves as fast as non-fragmented eggs, which shows that the potential on the heaviest fraction is close to -30 mv. This indicates that, when an egg is separated into three parts by the centrifugal force, the calcium layer is thrown down to the tiny heaviest part and stays there.

DISCUSSION

If the foregoing result is looked at from a different angle, we can interpret it as indicating that, when fragmentation of the cell occurs, the outer calcium gel is thrown to the centrifugal end, and that, if the fragmentation of the cell fails to happen, the sedimentation of

the outer layer also fails to occur. Now we face the question as to which one of the two is the cause and which one is the effect. At the present stage of our knowledge it is too premature to decide this point. The only fact available now is the fact that the absence of calcium in the medium shortens appreciably the time necessary for fragmentation. The writer has been following the line of thought that calcium ions solidify the outer layer, which is apparently a calcium gel of some sort. Then as far as the preceding piece of evidence is concerned, it adds some weight to the idea that it is this outer calcium layer which resists the tearing force and protects the eggs from fragmenting. Once it collapses, it snaps and shrinks to the centrifugal end, and the cell body is separated into two or three parts, as the case may be.

In the introductory part of this paper, I collected some facts indicating that in the centrifuged eggs there is a gradient of the membrane-forming capacity from the upper pole to the lower pole. As will be noticed immediately, this gradual transition in the membrane-forming capacity does not harmonize with the idea of a sudden collapse of the outer layer. I think these two things are mutually independent. The membrane-forming capacity is correlated with "the cortical layer" inside of the cell membrane. This is the cortical layer which Chambers (1921) and Just (1923) showed to be indispensable for the formation of the fertilization membrane. Independently of this, and on the outer side of the cell membrane, the calcium layer slides toward the heavy pole and the intervening cell membrane itself remains always intact. This must be the membrane which carries -20 mv., for, either after fragmentation by centrifuging or after the treatment with NaCl, we always find a surface characterized by the potential of -20 mv. spreading itself over the cell.

SUMMARY

1. The surface potentials of the centrifugal fragments of *Arbacia* eggs were studied.

2. The lighter halves have a potential of -20.9 ± 0.69 mv. and the heavier halves -27.6 ± 0.35 mv., while the centrifuged non-fragmented eggs have -30.8 ± 0.54 mv.

3. The results of the potential measurements indicate that the

rupture of the outermost layer (a calcium compound) occurs at the time of fragmentation.

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MOTILITY FACTORS IN MASS PHYSIOLOGY: LOCOMOTOR ACTIVITY OF FISHES UNDER CONDITIONS OF ISOLATION, HOMOTYPIC GROUPING, AND HETEROTYPIC GROUPING

(One figure)

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SOME of the most interesting of the phenomena exhibited by animal aggregations are the peculiar forms of physiological response of a group (or of a member of a group) of organisms in contradistinction to the characteristic response of an isolated individual of the same species to certain diverse physical or chemical agencies. The "group effect" may be displayed in one or more of the following ways: by differential growth rates (Adolph, 1931; Shaw, 1932), percentages of survival under unfavorable conditions (Allee and Bowen, 1932), reproductive rates (Park, 1932, 1933), differential sex ratios (Whitney, 1929; Brown and Banta, 1935), autotomy (Allee, 1927), conditioned learning times (Welty, 1934), oxygen consumption rates (Allee, 1927; Schuett, 1933), and other like quantitative criteria. These relationships existing in animal aggregations either in nature or in the confines of experimental media have been designated as phenomena of mass physiology (Allee, 1934).

In the case of oxygen utilization, Schuett (1933) reported experiments wherein the average oxygen consumption of each fish in a group of four goldfish placed in a given volume of water was less than the oxygen utilized by one fish confined to the same volume of water. Although he arrived at no definite conclusions concerning the reasons for the "group effect" in oxygen consumption, he was able to show by suitable running-water experiments that the decrease in oxygen tension of the medium was probably not responsible for the difference in oxygen consumption and that it was improbable that an

[1] We wish to acknowledge our indebtedness to Professors F. M. Baldwin and C. C. Lindegren for facilities and valuable suggestions.

accumulation of carbon dioxide or other metabolites played an important rôle in differential oxygen consumption. In a more recent paper Schuett (1934) states that the Winkler method for determining oxygen tension as used in his previous work has been found to be at fault and that in the light of new experiments no statistical significance can be ascribed to the small difference in oxygen consumption of grouped and isolated goldfishes as reported in 1933. By the use of quantitative methods Schuett (1934) found that less locomotor activity occurred in grouped goldfish than in isolated ones. It is thus reasonable to expect a real, even though small, difference in oxygen utilization in favor of isolated individual fishes unless some other, variable factor is present to neutralize the higher oxygen requirements entailed by their increased activity.

APPARATUS AND METHODS

For estimating the locomotor activity of fish we have devised two alternate methods, cinephotographic and observational, both depending upon the principle of plotting the spatial trajectory of the animal on three axes as a curve of closely successive points. This is made possible in both methods by optically subdividing the medium into small cubes which serve to locate the successive positions of the individual at very short intervals of time. An early version of this method has already been employed and described, with our consent, by Schuett (1934) in his studies on activity. An aquarium may be calibrated by painting parallel lines at suitable intervals on the walls of a rectangular glass aquarium, subdividing the surfaces into wide transparent stripes as follows: vertical stripes on the front wall for measuring movement on the length axis, horizontal stripes on the back wall for measuring movement on the depth axis, and vertical stripes on one end wall for measuring movement on the width axis. In order that all three sets of stripes may be viewed from the front and thus be photographed upon a single film when the cinematographic method is used, a plane mirror is placed near that end of the aquarium which lies opposite the calibrated end. Within a volume thus optically subdivided any given cube is in contact through its surfaces, edges, and corners with twenty-six adjoining cubes. There are, however, only three possible center-to-center dis-

tances between any cube and the contiguous ones. For example, in the case of cubes with a 2-cm. side, these distances are: 2 cm. for the six cubes in contact at the surfaces, 2.28 cm. for the cubes in contact at the twelve edges, and 3.46 cm. for the cubes in contact at the eight corners. Since the photographic method at once reveals the distance category of the cube into which the fish passes from an adjoining one, the total length of the trajectory is the sum of the distances between the cubes traversed. The error which arises from the fact that a fish does not proceed exactly from the center of one cube to the center of the next can be made negligible by diminishing the size of the cubes and employing as a reference point a small but distinct anatomical landmark on the fish. For example, the tip of the dorsal fin of the goldfish remains visible from all angles when 2-cm. stripes are used.

For the observational method the aquarium is calibrated as previously described. Three observers station themselves so that each observes the fish against one of the three striped walls of the aquarium, i.e., along one particular spatial axis. Each observer registers a unit on a hand tally when the fish passes one of the lines on the axis under his surveillance. The total number of stripes passed on the three axes is directly proportional to the extent of movement: the product of the width of the stripes and the number of lines passed approximates the true length of the trajectory. For accurate determinations a correction factor must be employed, since there is an inherent overestimation of the true length of the trajectory. The overestimation depends upon the shape of the trajectory and the width of the stripe used. The latter factor can be made small if the narrowest stripes allowing clear vision for the observers are used. Variations in the shape of trajectory may be corrected for as follows: A wire of known length bent into the shape of any hypothetical trajectory is placed within the calibrated aquarium. Then the length of wire is estimated by multiplying the width of the stripe by the total number of stripes that are seen to be "traversed" by the wire, as observed against the striped sides. When the percentage of overestimation (x) is found, a second wire, shorter by x per cent than the true length of the first, is bent into another hypothetical trajectory, placed in the aquarium, and estimated for length. The mean percentage of

overestimation obtained from several such attempts may be taken as the correction factor. For stripes 2 cm. wide, as used in the experiments reported here, the correction factor is approximately 1.4 per cent.[2]

For our experiments a rectangular glass jar 24 cm. in length, 12 cm. in width, and 24 cm. in depth was calibrated with stripes 2 cm. wide and used as an aquarium, being filled with water (from a deep artesian well) to 1,625 cc., 3,250 cc., 4,875 cc., and 6,500 cc. Isolated, and in groups of four, the activity of small specimens (6–8 cm.) of *Carassius auratus* L. was studied by daylight in a room maintained at 19.5°–20.5° C. Using the observational method, several 20-minute periods of measurement were carried out on successive days until twenty observations had been recorded for both conditions of aggregation in each of the four volumes of water. We have made similar but less extensive observations on the movement of 6–8 cm. specimens of *Carassius auratus* L., *Gambius affinis* L., *Oryzias latipes* Bleeker, and *Macropodus verdi-auratus* Lacapede, under conditions of isolation, homotypic groups of four, and heterotypic groups of four in which each fish was of a different species. The four species are those mentioned above. In the heterotypic groupings, care was taken to select individuals of approximately the same size. All animals, when not under observation, were maintained in aquaria in the laboratory in which the experiments took place. Four to 8 hours were allowed for the fish to recover from the effects of handling before the actual observations on activity were begun. At all times the observers followed the movements of the fishes from behind gauze curtains to preclude any reactions that the fishes might display due to motions on the part of the experimenters. The behavior of the fishes at no time indicated their detection of the presence of the observers.

DATA

Our experiments with *Carassius auratus* show that there is decidedly less locomotion when the fish under observation is a member of a homotypic group of four than when it alone occupies the same volume of water. This relationship holds good in each volume which

[2] The data given below are uncorrected.

we have tried, not only for mean values of the series of twenty observations (Table I), but also for most of the separate observations. Although there are within each series rather great variations from the mean, there are few overlapping values when data for the activity of isolated and grouped fishes are compared. The combined data for isolated and homotypic groups of *C. auratus* from Tables I and II are shown in Figure 1.

TABLE I

LOCOMOTION: MEAN VALUES OF TWENTY SEPARATE OBSERVATIONS IN EACH VOLUME AND AGGREGATION

Carassius auratus

Volume of Water (in Cc.)	Fish A		Fish B	
	Isolated (Meters per Hr.)	Grouped (Meters per Hr.)	Isolated (Meters per Hr.)	Grouped (Meters per Hr.)
1,625	83.40	17.26	103.40	39.10
3,250	83.44	55.47	69.43	39.65
4,875	51.33	32.54	61.95	13.08
6,500	118.18	58.43	100.09	50.94

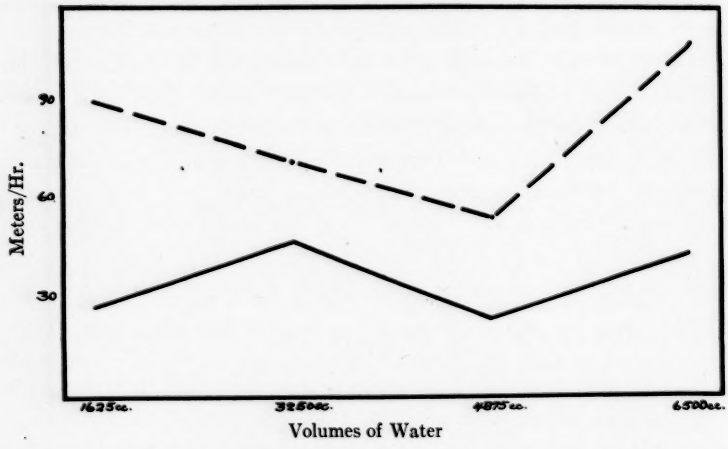

FIG. 1.—Dotted line represents movement of isolated *Carassius auratus;* solid line represents movement when in homotypic group of four. Mean values for forty-seven experiments each volume and condition of aggregation.

The activity of a solitary goldfish and of the same individual as a member of an homotypic group and as a member of an heterotypic group is summarized in Table II. These data suggest that, at least

TABLE II

LOCOMOTION: MEAN VALUES OF SEVEN OBSERVATIONS IN EACH VOLUME AND AGGREGATION

Volume of Water (in Cc.)	Isolated (Meters per Hr.)	Homotypic Group (4) (Meters per Hr.)	Heterotypic Group (4) (Meters per Hr.)
	Carassius auratus (Fish C)		
1,625	81.06	24.15	54.21
3,250	59.58	51.84	110.94
4,875	17.88	31.14	71.88
6,500	108.69	17.58	126.00
	Gambius affinis		
1,625	26.94	25.80	57.48
3,250	77.58	70.68	66.96
4,875	101.16	90.33	111.98
6,500	123.24	106.56	105.96
	Oryzias latipes		
1,625	27.96	39.54	75.84
3,250	75.30	82.11	97.08
4,875	65.64	51.54	80.40
6,500	111.98	104.34	105.84
	Macropodus verdi-auratus		
1,625	24.60	30.96	27.52
3,250	34.46	56.34	51.00
4,875	42.16	45.60	41.54
6,500	97.86	45.36	74.37

in the early stages of the artificial formation of such an heterotypic group, the several members display much more locomotor activity than in a comparable homotypic group, and slightly surpass the activity typical of the isolated individual. This observation, if subse-

quent confirmation should establish it as a constant phenomenon, might permit interpretations of sociogenic significance.

Of the other fishes studied (Table II), the activity of *Gambius affinis* most nearly ran the course described for *Carassius auratus*. The activity of *Oryzias latipes* and *Macropodus verdi-auratus*, particularly in regard to the comparatively small amounts by which the individual fishes in the heterotypic groupings excelled their own activity when in the homotypic groupings, might indicate variations in the response of the separate species to whatever integrative or dispersive stimuli exist in the respective aggregations. It is not beyond question, however, that such aberrations from the type of motility response occurring in *C. auratus* could arise from the differential effects which the temperature of the medium might exert on the various species.

Our data for the locomotor activity of isolated and homotypically grouped *Carassius auratus* and *Gambius affinis* are in agreement with the observations of Schuett (1934), who reported greater activity for isolated *C. auratus* than for groups of four in experimental volumes of 7,500 cc. and 15,000 cc. Although the plan of our experiments was such that the data derived from them do not lend themselves very well to testing Schuett's (1934) assertion that there exists an "optimal number" of individuals per unit volume of medium (i.e., that activity attains a minimum when a certain number of individuals are present), some comparison can be made between his results and ours. By far the greatest number of our observations (Table I and Fig. 1) show that minimal activity for both isolated and grouped goldfishes occurred at the same volume, namely, 4,875 cc. To this extent our experiments are not indicative of a relationship between "optimal number" of individuals and minimal activity for a given volume. Since we have dealt only with solitary fishes and groups of four, while Schuett studied, besides isolated individuals, groups of four, eight, and sixteen individuals, we cannot challenge directly his contention that an optimal number per unit volume holds wherein least activity occurs.

We were led to suspect that the spatial factors affecting total movement in different masses of water were of a more subtle nature than merely volume relationshops (i.e., total volume or volumes per

individual as expressed by previous workers). Statistical analyses of the data of 360 separate experiments show that the relative movement of fishes along the different axes of the aquarium (i.e., the particular length, width, and depth) bears a relationship to the magnitude of each of the three dimensions of the mass of liquid and only remotely to its volume. Combining the data for the goldfishes A and B in Table I, we have calculated "product-moment" correlations to show the relation between the total number of stripes traversed and

TABLE III

RELATION OF TOTAL MOVEMENT TO MOVEMENT ON ENVIRONMENTAL AXES*

Depth (in Cm.)	*rll*	P.E.$_r$	*rlw*	P.E.$_r$	*rld*	P.E.$_r$
	ISOLATED *Carassius auratus*					
5.64.....	+.995	.0107	−.213	.1030	+.437	.0875
11.28.....	+.816	.0360	+.168	.1050	+.212	.1031
16.92.....	+.232	.1021	+.195	.1040	+.416	.0893
22.56.....	+.175	.1049	+.381	.0923	+.700	.0551
	GROUPED *Carassius auratus*					
5.64.....	+.820	.0354	+.729	.0511	+.787	.0395
11.28.....	+.765	.0463	−.219	.1028	+.802	.0385
16.92.....	+.661	.0609	+.497	.0813	+.921	.0185
22.56.....	+.620	.0665	+.576	.0721	+.696	.0556

* *rll*=relation of movement on length axis to total movement. *rlw*=relation of movement on width axis to total movement. *rld*=relation of movement on depth axis to total movement. 1.00 denotes complete relation. P.E.$_r$=Probable error calculated from $[0.6745\times(1-r^2)]/\sqrt{N}$.

the number of stripes traversed on each axis. These data are given in summary in Table III.

In the case of the isolated fishes, it will be noted that the relation of movement on the length axis to total movement (*rll*) is highest when the depth axis is most restricted, and that it diminishes as the depth increases. Since 1.00 denotes perfect relation, the correlation +.995 indicates that a very great portion of the total locomotion recorded in each experiment occurred on the length axis. A correlation below +.900 indicates a moderate relationship; less than +.700 de-

notes more or less random relationships. The values of *td* tend to increase in the same order as the increase of the depth axis, indicating that this dimension has become available for movement. The data for the relation of locomotion on the width axis to total locomotion (*rtw*) show that random movements prevail on this axis throughout.

For the grouped fishes, our data reveal no correspondingly high correlations under similar conditions; nor do movements seem to be at random to so great an extent. A member of a group does not tend to move along any particular axis so consistently. Whatever may be the influence of the magnitude of any dimension, it is apparently less powerful in the group. If one should arrange a container of such dimensions that locomotion would be unrestricted only on one dimension, one would expect a high relation between total movement and locomotion on the unrestricted axis. A fish 6 cm. in length, such as used in our experiments, placed in an aquarium 500 cm. long, 4 cm. wide, and 4 cm. deep, would be limited to movements along the length axis and might indeed experience some difficulty in reversing its course. On the other hand, little difference in degree of influence might be expected if the aquarium were a cube with a 20-cm. side, having the same volume as the former.

Furthermore, if the 500-cm.-long container described above were stood on end so that its long axis were vertical, a fish placed in it would have at its disposal a depth of 500 cm. of water for movement, without being able to move freely along the dimensions of width and length of the container. These extreme examples of spatial restriction serve to emphasize the influence that magnitude of environmental dimensions can exert on the motility of fishes.

In the previously described vessel one would expect an inclosed fish to move more freely, and hence possibly more per unit time, with the container having its long axis in the horizontal position rather than in the vertical. A priori, such behavior is expected in view of the fact that the locomotor mechanisms of fishes (with a few exceptions, e.g., seahorse) are adapted to propel the fish along the long axis of its body, the latter being normally oriented in most species of fishes in a horizontal plane. In experiments on fishes, the shape of the aquaria is usually of the form of a rectangular parallel-

opiped, in which no one dimension differs greatly from the other two. In an aquarium of this shape the horizontal components of the movement of a fish usually exceed the simultaneous vertical components of that movement.

We are inclined to believe that when a fish is limited to a given volume of water the configuration of that volume, or the interrelation between the relative magnitudes of its three dimensions, determines to a considerable extent the total amount of movement.

In view of the preceding considerations an explanation may be advanced for our finding that minimal activity in both grouped and isolated goldfishes occurred at a volume of 4,875 cc. having a depth of 16.92 cm., a width of 12 cm., and a length of 24 cm. The latter two dimensions are constant for the four volumes of water used, since they are the dimensions of the container, the depth axis alone varying with increasing volume. This is shown in Table III.

The fact of the occurrence of least locomotor activity for grouped, as well as for isolated, fishes at a volume of 4,875 cc. may possibly be attributed to the coincidence that at this particular volume and for the size of fishes used there occurred a relation between the dimensions which makes for diminished activity. It appears that the spatial characteristics of the medium other than volume influence the activity of fishes. The several axes or dimensions of the mass of water, and possibly its configuration as well, exert effects. So many variables impinge upon analyses of animal movement in tridimensional space, especially when complicated with the volume available for movement, that at present one can merely recognize certain physical and biological characteristics of the organism which are likely to interplay with the spatial characteristics of the medium to yield a specific quantity of movement.

Degree and type of somatic axiation, degree of cephalization, normal orientation in space, methods of propulsion, specific gravity, susceptibilities to geotropism, to thigmotropism, and degree of irritability to repeated contacts, etc., are biological peculiarities which probably influence the kind and extent of movement performed in restricted masses of water of diverse configuration.

The possibility that certain spatial factors other than volume may influence the behavior of active animals restricted to small vol-

umes of media is suggested by the experiments of Bilski (1921), who found that the growth of tadpoles was affected by the relative size of the vessel, the volume per individual being kept constant. Bilski believed that growth was retarded by the stimulation from contact or near approach of two individuals and that possibly such contacts were in proportion to the sizes of containers. Thus for two containers, a and b, having an equal density of population, the probabilities of intragroup stimulation would be $a(a-1)$ and $b(b-1)$. If the relation of the size of the containers is taken as 2:3, the calculated probabilities of stimulation are 2:6.

Why an isolated fish moves more than a member of a group (at least in small containers) is a still more obscure phenomenon. After having examined motion-picture films of both isolated and grouped fishes, it is our belief that two types of behavior displayed may yield a clue to possible explanation. First, it appears that the "free path" or direction of movement of a grouped fish is being continuously obstructed by the nearby presence of his fellows. Our records show that a solitary fish describes a path or trajectory that is more smooth and much more free from reversals than the path of a member of the group. Furthermore, cinephotographic records show that members of a group are continuously making small adjustments in their orientation or movements as they react to the presence of their nearby fellows. From the examination of our motion-picture films it appears that the total movement performed during a given time interval is always less when the separate movements are short and interrupted by pauses than when they are more continuously prolonged as in the case of the solitary fishes.

Of these reactions, just what is cause and what effect remains to be determined.

It becomes increasingly obvious that, in order to obtain comparable data, investigators dealing with problems of locomotor activity must adhere to strictly controlled experimental conditions. The necessity of standard temperature, reaction, and chemical composition of media is widely recognized. It would seem justifiable to expect equal exactitude in the selection of individuals of known age and size, inasmuch as locomotor ability is closely related to chronological changes in corporal development. Particularly in experiments with

homotypic groups, the sex of adult members becomes significant in determining the behavior of the individual. Differential locomotor activity in the sexes has recently been demonstrated by Slonaker (1935) in the rat. Even in immature individuals sex cannot be ignored, since there may exist dimorphisms and differential physiological abilities. The results of investigations upon activity differences between the two homosexual homotypic groups of animals are certain to prove both interesting and sociologically significant.

In the case of animals with highly developed visual sense organs, the contour and coloration of their fellows in homotypic or heterotypic groups may conceivably alter their movements, resulting in various degrees of aggregation or dispersal.

Especially in view of the fact that the integration of certain aggregations of fishes has been demonstrated by Bowen (1931) to be visual, it becomes imperative to carry all observations on the activity of fishes under conditions of symmetrical and constant illumination. Only in this way will it be possible for even the same investigators to obtain reproducible results. Furthermore, certain experiments performed in this laboratory (unpublished) impress upon us the absolute necessity of a constant artificial illumination.

The very nature of the phenomenon of animal movement, bristling as it does with intangible and uncontrollable variables, requires from the early investigator a most scrupulous study entailing a thorough control of all the recognized factors involved.

SUMMARY

1. Cinephotographic and observational methods for estimating the locomotor activity of animals in tridimensional space have been described.

2. In *Carassius auratus*, the activity of an isolated individual is greater than the activity of the same individual when grouped with three other goldfishes. The activity of the goldfish when as a member of an heterotypic group exceeds even that in the isolated condition. The behavior of *Gambius affinis*, *Oryzias latipes*, and *Macropodus verdi-auratus* in isolated and aggregated conditions is reported.

3. Certain suggestions concerning the selection, conditioning of experimental animals, and the control of the environment in activity studies have been made.

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THE DIFFERENTIAL REACTION OF THE BLOOD VESSELS OF A BRANCHIAL ARCH OF AMBLYSTOMA TIGRINUM (COLORADO AXOLOTL) I. THE REACTION TO ADRENALIN, OXYGEN, AND CARBON DIOXIDE[1]

(Three figures)

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DURING a study of the problem of gill reduction in amphibians, the hypothesis that the process of gill reduction is closely related to the reactions of the branchial vessels suggested itself. A study of the reactions of the branchial vessels in *Amblystoma tigrinum* larvae was attempted, therefore, in the hope that this would either confirm or disprove the foregoing hypothesis. In addition, it was thought that such a study might add a few facts to our knowledge of the general physiology of blood vessels.

The present paper will deal with the reaction of the branchial vessels to adrenalin, oxygen, and carbon dioxide. Evidence will be presented to show that there are two sets of blood vessels in the same gill arch, one set of which dilates under the influence of adrenalin, while the other set constricts. This reaction, hereafter, will be referred to as a "differential reaction" of the blood vessels of a branchial arch.

It had been stated in earlier papers (Figge, 1930, 1934) that a change in the oxygen tension of the blood supplying the gills was at least partly responsible for gill reduction. It was, therefore, especially desirable to know how these two sets of vessels would react to changes in the oxygen and carbon dioxide tension of the blood. It was found that the two sets of blood vessels also reacted in a differential manner to both oxygen and carbon dioxide.

[1] This paper is part of a thesis submitted to the faculty of the University of Maryland Graduate School in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

For the sake of clarity and to facilitate the accurate description of the details of the physiological reactions, a brief description of a diagram of a typical branchial arch of *Amblystoma tigrinum* and a discussion of the hydrodynamics will follow.

THE BLOOD VESSELS OF A TYPICAL BRANCHIAL ARCH IN *Amblystoma tigrinum* LARVAE

The blood vessels in the branchial arches may be divided into two groups: those forming the aortic arch proper, or primary circuit (see

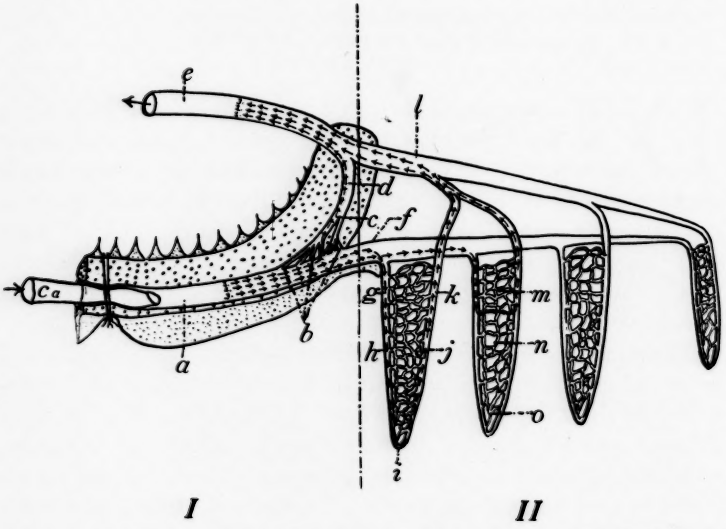

FIG. 1.—A diagram of a cannulated branchial arch. I, anastomotic portion; II, gill proper; *a*, ventral portion of the aortic arch; *b*, anastomotic arterioles; *c*, *d*, intermediate anastomotic vessel; *e*, dorsal portion of aortic arch; *f*, afferent branchial artery; *g*, *h*, afferent limb of the arteriole loop; *i*, arteriole at tip of the filament; *j*, *k*, efferent limb of the arteriole loop; *l*, efferent branchial artery; *m*, *n*, *o*, capillary bed of filament; *Ca*, cannula; heavy stipple, visceral cartilage; fine stipple, gill blade. The three groups of arrows indicate three possible routes which blood may take in passing from the ventral to the dorsal aorta.

Fig. 1); and those making up the gill proper, or secondary circuit. The aortic arch or primary circuit consists of three parts: a large ventral vessel, *a;* a large dorsal vessel, *e;* and intermediate anasto-

motic vessels, *b*, *c*, *d*. The gill, or secondary circuit, is composed of the afferent branchial artery, *f;* the arterioles, *g*, *h*, *i*, *j*, *k;* the capillaries, *m*, *n*, *o;* and the efferent branchial artery, *l*.

From the diagram (Fig. 1), it is evident that there are three ways for blood to pass from the heart to the dorsal aorta:

1. It may pass through the ventral portion of the aortic arch, *a*, and thence through the intermediate anastomosis, *b*, *c*, *d*, to the dorsal portion of the aortic arch, *e*.

2. It may pass from *a* to the afferent branchial artery, *f*, and thence through the arteriole, *g*, *h*, *i*, *j*, *k*, to the efferent branchial vessel, *l*, which communicates with *e*, the dorsal portion of the aortic arch.

3. Or it may pass through *f* to the afferent limb of the arteriole and then through any capillary in the capillary bed, *m*, *n*, *o*, to the efferent limb of the arteriole, and thence to *l* and *e*.

Under normal conditions in the larval animal some blood travels from the heart to the dorsal aorta by all three of these routes. In order for any blood to flow through the capillary bed, *m*, *n*, *o*, of the filament, the pressure must be greater in the afferent limb of the arteriole, *g*, *h*, than it is in the efferent limb, *j*, *k*.

The morphological conditions that make possible the differences in pressure in the two limbs of the arteriole are as follows: both the efferent and afferent limbs of the arteriole loop of the filament taper toward the tip, where they communicate directly with each other, so that at point *i*, the diameter of the arteriole is much less than at either *g* or *k*. It is evident that if a liquid was forced through this arteriole from *g* to *k*, the pressure would be greater at *g* and *h* than at *j* and *k*. Then, in addition to the tapering of the afferent limb of the arteriole, the viscosity of the blood and the fact that the arteriole is bent upon itself, tend to make the pressure lower in the efferent limb, *j*, *k*, than in the afferent limb, *g*, *h*.

By far the most important mechanism which controls the difference in pressure in the two limbs of the arteriole loop is the anastomosis, *b*, at the base of the gill. As long as the vessels making up the anastomosis have a small diameter, the pressure in the dorsal portion of the aortic arch, *e*, and the efferent branchial artery, *l*, may be much lower than the pressure in the ventral portion of the aortic

arch and the afferent branchial vessel. If the vessels making up the anastomosis at *b* dilate, there is a tendency to increase the pressure at *e*, *l*, and *k*, and, at the same time, to decrease it at *a*, *f*, and *g*. Dilatation of the anastomotic vessels, *b*, has a pronounced tendency to equalize the pressures in both limbs of the arteriole loop of the filament, and thus to stop the flow of blood through the filaments.

There are, then, three factors that regulate the flow of blood through the capillaries in the gill filament. One is the tone or diameter of the capillaries themselves (*m*, *n*, *o*); another is the tone or diameter of the arteriole at the tip of the filament, *i;* and last, but not least, there is the tone or diameter of the arterioles in the anastomosis at the base of the gill.

METHODS AND MATERIALS

The animals used were normal *Amblystoma tigrinum* larvae (Colorado axolotls), one to three years old (body length, 70–140 mm.). All animals were kept in individual glass bowls of 2-liter capacity. They were fed beef liver twice weekly; occasionally earthworms were subsituted for the liver.

METHODS AND MATERIALS FOR PERFUSION EXPERIMENTS

The animals were anesthetized in 0.1 per cent *chloretone* solution previous to the perfusion of all gills or the perfusion of an isolated gill. Unless otherwise stated, the perfusion was carried out at constant pressure of 250 mm. of water. This is slightly higher than the normal blood pressure. In order to maintain a constant pressure and determine the rate of inflow, the following method was devised: The cannula was connected, by means of rubber tubing and a T-tube for a bubble-catcher, to the bottom of a test tube. The upper end of the test tube was stoppered with a one-hole stopper, into which a 6-inch length of 8-mm. glass tubing was placed. This entire system was filled with perfusion fluid up to a mark near the middle of the 8-mm. glass tube. A burette was arranged so that the tip drained into the upper end of the glass tube. The burette was constantly adjusted, so that as fast as the perfusion fluid flowed out through the gill preparation it was replaced by fluid from the burette. The burette readings were taken at short intervals, and the amount of fluid necessary

to maintain a constant level in the tube was calculated from these. The results were then plotted from these calculations.

The gill preparations were immersed in tap water in a wax-bottom dish. The perfusion fluid was allowed to flow through the gill preparation out into the tap water through the open ends of severed blood vessels. A continuous stream of tap water was passed through the dish. This was done to maintain a constant temperature 20°–22° C. and to prevent the accumulation of adrenalin in the external medium. The preparations were tested for leaks at the end of the experiments by placing a dye in the perfusion fluid. The slightest leak could be detected by observing the outflow. A leak was found in only a few cases, and these results were discarded. The capacity of the cannula at the pressure used was measured in each case to make certain that this was much greater than the capacity of the gill that was being perfused.

In preliminary experiments all six gills of one animal were perfused simultaneously by cannulating the truncus arteriosus. The perfusion fluid was allowed to flow out of the cut ends of the dorsal aorta and the carotid arteries.

In another preliminary experiment, a single gill arch was perfused (see Fig. 1). This was removed from the animal by ligating the aortic arch ventrally as near the heart as possible, and dorsally as near the radix aortae as possible. The ventral portion of the aortic arch, *a*, was then cannulated. The perfusion fluid thus passed through either the gill or the anastomosis to reach the outflow at the cut end of the dorsal portion of the arch *e*.

To perfuse an isolated gill (without the anastomosis), the external gill was cut off just distal to the anastomosis (see point *f*, Fig. 1). The afferent branchial artery, *f*, was then split for a distance of about 2 mm. to facilitate cannulation. As soon as this vessel was cannulated, it was tied in place by a double ligature. The perfusion fluid was allowed to slowly escape from the cannula during the cannulation. The whole process of cannulation was carried out in a wax-bottom dish filled with perfusion fluid. As soon as the ligatures were tied, the full perfusion pressure was turned on. The perfusion fluid escaped through the cut end of the efferent branchial artery, *l*, In order to reach the outflow vessel, the perfusion fluid had to pass

through the gill filaments. Two isolated gills were perfused simultaneously, one with perfusion fluid alone, the other with perfusion fluid plus adrenalin. After a certain time, the two tubes were changed from one cannula to the other, so that the one that had been perfused with perfusion fluid alone was, after the exchange of tubes, perfused with perfusion fluid plus adrenalin, and vice versa.

The technique used in perfusing an isolated anastomosis was as follows (see Fig. 1): The whole gill arch was removed, as described above. The gill was severed from the visceral arch just distal to the anastomosis, *b*, leaving only a few filaments on the visceral arch portion. The ventral end of the aortic arch, *a*, was then cannulated. Any blood contained in the vessels was forced out before the afferent branchial artery, *f*, was ligated just distal to the anastomotic vessels. All the filaments on the preparation were included in the ligature. The perfusion fluid was allowed to flow out of the cut ends of the efferent branchial vessel, *e* (see Fig. 1).

The perfusion fluid employed was a modified Ringer-Locke solution that was made up fresh each day. The concentrations of the various salts were adjusted to produce a solution that was isotonic with larval salamander blood. The osmotic pressures of the perfusion fluid and of larval salamander blood were checked by the cryoscopic method. Both perfusion fluid and larval blood showed a freezing-point depression of 0.468° C. The perfusion fluid, as used, contained the following substances per liter of water: NaCl, 6.5800 gm.; KCl, 0.2650 gm.; $CaCl_2$, 0.2004 gm.; $NaHCO_3$, 0.4 gm.; dextrose, 0.9 gm.; urea, 0.5 gm.

The adrenalin chloride (1 : 1000) was obtained from Parke, Davis and Company. Adrenalin solutions were prepared just before use.

METHODS AND MATERIALS FOR DIRECT OBSERVATION EXPERIMENTS

To confirm the results obtained by perfusion, direct observations were made on the branchial vessels of the intact animal. In order to do this, the animal was kept in shallow water and a continuous stream of water was passed through the dish. The temperature was kept at 20°–22° C. This was found to be an important factor, since the temperature, more than anything else, regulates the heart rate. The heart rate was checked before and after many experiments

to make certain that this factor was not responsible for the changes observed in the gill vessels.

The anesthesia used to immobilize the animal for making direct observations was, in most cases, nembutal, the dose depending on the size of the animal. This was injected subcutaneously in the midline just caudal to the gills, or, in other words, into the most cephalad part of the dorsal body fin. Nembutal was found to be far superior to chloretone, urethane, chloroform, or ether. All of the last-mentioned drugs, in concentrations sufficient to produce anesthesia, cause such great variation in heart rate and vascular tone that it was impossible to work with them. Chloretone anesthesia very frequently stops the flow of blood in the gills almost completely; this is due not to the constriction of gill vessels but to a very low heart rate. With nembutal, however, the circulation was always very good in all parts examined, and the heart rate varied only with variations in temperature. After some experimentation with the dosage, it was found possible to regulate this according to the size of the animal and the length of the anesthetic period desired. It was possible, with nembutal anesthesia, to make observations on the branchial vessels for as long a period as 10–15 hours. The animals usually recovered within 24 hours. A few curarized animals were used. In order to avoid the possible criticism that the effect observed was due to the anesthesia, a number of experiments were performed on unanesthetized animals. In the latter case, the animal was placed in a condom. This was then pinned down at various places in a wax-bottom dissecting dish. The animal was almost completely immobilized in this way and struggled very little after the first few minutes. A slit was made in the condom near the base of the animal's gills, and these were pulled out through the slit.

By placing a gill or an individual filament between a reflector (or white piece of paper) and the objective of a binocular dissecting microscope, it was possible to determine changes in the diameter of any vessel in the gill filament, even without the aid of artificial light. The changes in the diameters of the gill vessels were determined by means of an ocular micrometer. Three to five control observations were made before the application or injections of adrenalin. After the application or injection an equal number of observations were made

at various intervals. Many observations were made without anesthesia and without artificial light, but in most cases an arc light was used to facilitate the observations. The light from this was cooled and filtered by passing it through thick glass and a light-blue solution of copper sulphate. This light had no detectable effect on the vessels.

It was found possible to place one of the animal's gills in a special observation dish or tray. The small dishes or trays were trough-shaped and were made either of glass or aluminum foil. They were made just large enough to hold one gill and a small amount of the solution to be tested. The animal was pinned down in a wax-bottom dish so that the gills on one side floated near the surface of the water in the dish. One of the gills was then placed in the small tray. By this means one gill was isolated from all the other gills. The edge of the small tray over which the gill passed was fashioned out of Plasticine to conform to the shape of the gill. This prevented the fluid in the small tray from mixing with that in the large dish in which the animal and control gills were immersed. In addition, it was not necessary for the gill to bend over an edge to lie in the small tray. Any disturbance of the blood pressure or blood flow in the test gill due to bending of gill vessels over the edge of a tray was thereby avoided. Any gill could thus be immersed in solutions containing any test substance in any desired concentration. The corresponding gill on the other side was used as a control gill.

Eventually a technique was devised which permitted the application of a test substance to a single gill filament. The animal was likewise pinned down, ventral side up, in the wax-bottom dish so that the gills on one side floated near the surface. One of the gills was then supported by a wax-covered white paper. Many of the filaments were thus caused to lie flat on the wax surface. By scooping out the wax under these filaments, small pockets were made in the surface of the wax, in which the individual gill filaments rested. When small amounts of adrenalin were applied to these individual filaments, these pockets inhibited the diffusion of adrenalin to other filaments. It was thus possible to observe the effect of adrenalin solution on the blood vessels of a single gill filament. The nearby filaments were regarded as controls and observed simultaneously.

METHOD OF REGULATING THE O_2 AND CO_2 TENSION OF THE BLOOD SENT TO THE GILLS

After the animal was anesthetized or immobilized as previously described, it was pinned down in a wax-bottom dish partly filled with salamander Locke-Ringer solution. An incision was then made in the abdominal wall just lateral to the estimated position of the tip of the lung, which was pulled out and cannulated. The lung was then pushed back into the abdominal cavity to near the normal position. The cannula was kept in position by a holder that rested on the wax in the dish. It was arranged so that either a stream of oxygen or carbon dioxide or any desired mixture of these gases might be passed through the cannula. The gas which passed through the lungs was driven out, either through the glottis or through a hole in the tip of the other lung.

The humidification of the test gases was effected by bubbling them through distilled water. Another bottle with a longer inlet tube and a higher column of water served as a pressure-regulator or safety valve, so that it was not possible for the pressure in the cannula to go above 6 cm. of water.

The effect of the variation in the oxygen and carbon dioxide tension in the blood was determined by direct observation on the gill vessels with the aid of a binocular dissecting microscope and filtered light, as described above.

RESULTS

A. THE EFFECT OF ADRENALIN ON THE BRANCHIAL VESSELS AS DETERMINED BY THE PERFUSION METHOD

a) *Perfusion of all gill arches simultaneously.*—In preliminary experiments the method employed by Keys and Bateman on eel gills, and Krawkow on fish gills, of perfusing all gills at once, was tried. It was found in these experiments that the rate of perfusion was not dependent entirely on the size of the vessels perfused but was influenced by the rhythmic contractions of the bulbus arteriosus. Moreover, the rate of perfusion did not change appreciably on addition of adrenalin. These negative results suggested that one set of vessels in the branchial circulation reacted by dilatation with adrenalin while another constricted. Because of this, the perfusion of a single gill arch and part of a gill arch was attempted.

b) *Perfusion of a single gill arch.*—In another preliminary experiment, a whole gill arch (external gill plus the anastomosis at the base) was perfused (see Fig. 1). A ligature was placed around the anastomotic vessel at *d* so that it could be tightened or released at will. During a control period of 40 minutes, the rate of perfusion for the entire branchial arch averaged 23 cc. per hour. When the ligature was tightened and tied securely, only the external gill or gill proper was perfused. The perfusion rate fell to 3 or 4 cc. per hour. Adrenalin was added to the perfusion fluid, and the rate of perfusion promptly increased to 15 cc. per hour. This rate was maintained for about an hour; then the gill was perfused with perfusion fluid alone. The rate of perfusion gradually fell to 6 cc. per hour. The ligature was then removed from the anastomotic vessel, and the entire branchial arch was now perfused. The rate of perfusion increased to 30 cc. per hour. Adrenalin was then added to the perfusion fluid; and this had little, if any, effect. From this preliminary experiment it was evident that, in order to test the effect of any substance on the gill vessels, it would be necessary to perfuse the gill proper and the anastomosis separately.

c) *Perfusion of the isolated external gill or gill proper* (without the anastomosis).—Adrenalin was used in concentrations ranging from 1:50,000 to 1:5,000,000. All were found effective and had the same action. It was tried seventy-two times on thirty-five individual gills. The gills were cannulated distal to the anastomotic vessels (see point *f*). In every case except one, the addition of adrenalin to the perfusion fluid increased the rate of perfusion. In this case, perfusion fluid a week old had been used.

The effect of the addition of adrenalin to the perfusion fluid may be described more quantitatively by the use of graphs (see Fig. 2, gills *12B* and *13B*). The results obtained from two gills that were perfused at the same time are plotted on the same paper. It will be seen that, when a gill that had been perfused with perfusion fluid alone was perfused with perfusion fluid plus adrenalin (1:1,000,000) there was a distinct increase in the rate of flow. Sometimes the increase was as great as 200 per cent. This gill was again perfused with perfusion fluid alone, and the rate of perfusion dropped to the original level.

d) *Perfusion of the isolated anastomosis.*—When the anastomosis at the base of a gill was isolated and perfused by the technique described, the detached gill was placed on another cannula and perfused at the same time (see Fig. 1). In order to compare the reactions, the rates of perfusion were plotted in the same graph (see

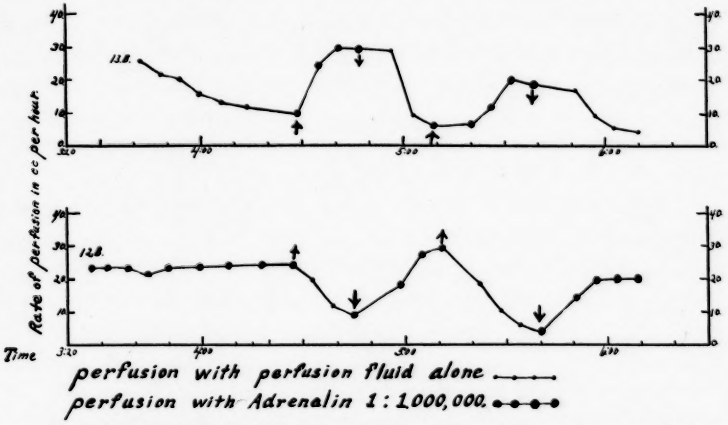

FIG. 2.—The quantitative effect of adrenalin on the perfusion rate in isolated gills (see Fig. 1). *12B*, right second gill; *13B*, right third gill. Arrows indicate the beginning and termination of periods during which the preparations were perfused with perfusion fluid plus adrenalin. These graphs show, quantitatively, that the addition of adrenalin to the perfusion fluid during the perfusion of isolated external gills causes the perfusion rate to increase.

Fig. 3). It was observed that the reaction of the anastomotic vessels to adrenalin is just opposite to that observed in the gill. When adrenalin was added to the perfusion fluid, the arterioles making up the anastomosis constricted and caused a decrease in the rate of perfusion. The effect was not as marked as the dilatation was in the case with the isolated gill but was, nevertheless, quite distinct.

The results of perfusion of a first gill and corresponding anastomosis may be seen quantitatively in Figure 3 (gill *10A* and *23B*). The curve *23B* represents the rate of perfusion in the gill proper of the first branchial arch. As stated previously for the isolated gill, perfusion with the perfusion fluid plus adrenalin (1:50,000) caused an increase in the rate of perfusion; while perfusion with perfusion

fluid alone caused a marked decrease in the rate of perfusion. The rate with adrenalin in this case was 50–60 cc. per hour. When perfused with perfusion fluid alone, the rate dropped to 34 cc. per hour.

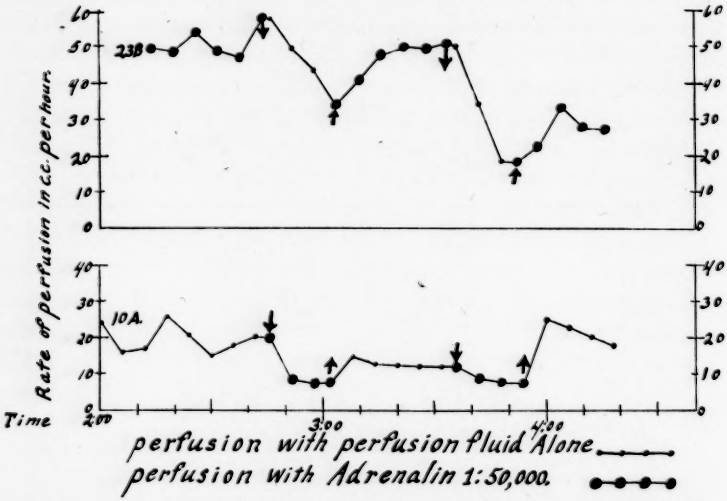

FIG. 3.—A comparison of the effect of adrenalin (1:50,000) on the rate of perfusion in an isolated gill proper (*above*) and in an isolated gill arch anastomosis (*below*) (see Fig. 1). Arrows indicate the beginning and the termination of periods during which the preparations were perfused with perfusion fluid plus adrenalin.

The isolated anastomosis was perfused at first with perfusion fluid alone, while the isolated gill was perfused with perfusion fluid plus adrenalin. Then the tubes were exchanged on the cannulae. The anastomosis was now perfused with perfusion fluid plus adrenalin, which constricted the anastomotic vessels, as evidenced by the decrease in the rate of perfusion. Meanwhile, the gill was perfused with perfusion fluid alone. This (as has already been demonstrated) caused a decrease in the perfusion rate of the gill.

The two perfusion tubes were again exchanged on the cannulae. The gill was now perfused with adrenalin, while the anastomosis was perfused with perfusion fluid alone. The rate of perfusion increased in both preparations. Then the same procedure was repeated with similar results.

It is evident from this that adrenalin constricts the anastomotic vessels at the base of the gill, while it dilates the vessels in the gill proper of the same gill arch.

Adrenalin solution was again perfused, and the rate went up to 50 cc. per hour. A change to perfusion fluid alone caused a decrease in the rate of flow to 18 cc. per hour.

During the time that the gill proper was being perfused with adrenalin, the anastomosis from the same branchial arch was being perfused with perfusion fluid alone. When the tubes were exchanged on the cannulae, the gill was then perfused with perfusion fluid alone, and the anastomosis with adrenalin solution. The reaction of the anastomotic vessels to adrenalin was just the opposite to that of the gill. The gill vessels constricted when they were perfused with perfusion fluid alone, while the anastomotic vessels constricted when they were perfused with adrenalin solution. The rate of perfusion in the anastomosis during a 50-minute period averaged about 20 cc. per hour (see Fig. 3). When perfused with adrenalin solution, the rate dropped to 7.5 cc. per hour. A change to perfusion fluid alone increased the rate to 15 cc. per hour. The rate of perfusion then gradually decreased to 12 cc. per hour. When adrenalin was again perfused, it fell to 7.5 cc. per hour. Perfusion without adrenalin solution then increased the rate to over 20 cc. per hour.

The experiment was repeated forty times on twenty-two gill anastomoses. The concentration of adrenalin ranged from 1:10,000 to 1:5,000,000. The anastomoses from all aortic arches were tested. Only five anastomoses did not respond to adrenalin by constriction. Three of these were found to have a leak in the system. One was perfused with perfusion fluid a week old. Why there was no response in the one anastomosis is not known.

B. THE EFFECT OF ADRENALIN ON THE BRANCHIAL VESSELS AS DETERMINED BY DIRECT OBSERVATIONS

It is evident from the perfusion experiments that adrenalin constricts the arterioles in the anastomosis at the base of the gill, while it dilates the vessels in the gill itself. It was impossible to determine just which vessels within the gill proper dilated when perfused with adrenalin. To confirm the results of the perfusion experiments and, in addition, to determine which vessels in the gill reacted to adrenalin, direct observations were made on the branchial vessels of the intact animal.

The effect of adrenalin was tested thirty times on twelve different animals. The concentrations ranged from 1:2,000 to 1:1,000,000. The effect observed was always an unmistakable dilatation of the capillaries of the gill filaments. In addition to the observations that

the capillaries increase in diameter, there is other evidence in favor of a capillary dilatation.

While observing the capillaries in the tip of a gill filament, 0.2 cc. of a 1:3,000 solution of adrenalin was injected into one of the aortic arches. Strangely enough, the blood stopped flowing through the vessels at the tips of the filaments (see point *o*, Fig. 1). That this was not due to constriction of the vessels was evident. Moreover, the vessels at the tip were filled with blood which fluctuated back and forth with each heart beat. Why the blood should stop flowing through the tip of the filament was indeed perplexing. Later it was observed that the capillaries at the base of the filament had dilated to such a degree that it was possible for all the blood to pass through a few capillaries located near the base of the filament (see *m*, Fig. 1). Here it flowed quite rapidly, following the easiest and shortest route through the filament.

In addition to the microscopic observations, there is macroscopic evidence in favor of dilatation of branchial vessels. The intravenous injection of adrenalin, or the immersion of a gill in adrenalin solutions, causes the gill filaments to increase in size. The whole gill becomes distended and reddish in appearance. In one case the increase in length of a single filament was measured. Before adrenalin, it measured 2.5 mm.; afterward it measured 3.5 mm. Since each filament is composed mostly of capillaries, the increase in size may be taken as evidence in favor of capillary dilatation.

By the method of direct observation, it was possible to show conclusively that adrenalin, in all concentrations tested, produced an extreme dilatation of the gill capillaries.

C. THE REACTION OF THE BRANCHIAL VESSELS TO O_2 AND CO_2 AS DETERMINED BY DIRECT OBSERVATION

The effect of oxygenated blood on the branchial vessels was tested thirty-five times on ten animals. The result was, in all cases, a decrease in the amount of blood passing through the gill capillaries. If the oxygen treatment was continued long enough, a complete cessation of the flow of blood in the gill capillaries resulted. This was not due to a decreased heart rate. The heart rate was taken before and during the administration of oxygen; and it was found that usually it was the same during the treatment with oxygen, occasionally less,

and sometimes even higher. Moreover, it was noted that, even though no blood flowed through the gill capillaries, it was flowing very rapidly through the capillaries in the gill blade, operculum, and the lung. The cessation of the flow of blood in the gill capillaries cannot, therefore, be due to a decrease in the heart rate or general blood pressure.

The direct observation of these vessels shows that the arteriole loop of the filament dilates in both limbs and especially at the tip (see Fig. 1). One is also able to observe an active constriction of the capillaries. Both of these reactions are difficult to describe quantitatively; but in addition to the observations on the change in diameter of the capillaries, there are other observations that may be taken as evidence of active constriction. If the capillaries were merely passive and had no way of increasing or decreasing their tone, the only factor that would cause a cessation of the flow of blood in them would be an equalization of pressures at both ends of the capillary, a condition that might be the result of a dilatation of the anastomotic arterioles, *b*. That this is not the only factor operating to stop the flow of blood in the capillaries may be seen from these facts (see Fig. 1):

1. The pressures on both ends of the capillaries are never equal as long as blood flows through the arteriole, *g*, *h*, *i*, *j*, to *k*. For, if the pressure at *g* were exactly equal to that at *k*, no blood would flow from one point to the other. The capillaries were frequently closed off while blood flowed through the arteriole loop from *g* to *k*.

2. If it were merely a matter of equalization of pressures at both ends of a capillary, we would expect all of them in one filament to close off at once. But this did not happen. In most cases, the capillaries at the base *m*, or middle *n*, of the filament closed off first, and later the capillaries at the tip of the filament. Sometimes, however, it was just the opposite. At any rate, blood did not stop flowing in all of them at once. When the lungs were ventilated with carbon dioxide and blood again began to flow through the capillaries in the filament, the last capillaries to close down were the first to open up. Different capillaries thus show differences in threshold.

We may conclude from this that there is an active constriction of the capillaries in the filament when the filament is supplied with oxygenated blood.

A portion of a protocol of one of these experiments follows:

Animal XXVI B 6, Colorado axolotl, curarized, January 13, 1934. Condition: Larval, normal. Respiratory reflexes (as evidenced by occasional gill movements) intact during the entire experiment.

3:40–4:20 P.M.	Operation: An incision made in right side, tip of lung pulled out and cannulated. Left lung was likewise pulled out and cannulated to allow the gas to escape from the tip of that lung.
4:25 P.M.	Pulse rate: 57 beats in 60 sec.
4:28 P.M.	Pure oxygen started through lungs. Bubbles coming out of animal's mouth and incision in left lung.
4:33 P.M.	Many of the capillaries in gill filaments closing off. Heart beating as fast as ever. Respiratory movements cease. Filaments seem to be decreasing in size. Heart rate: 45 beats in 47 sec.
4:35 P.M.	Gill capillaries, *m*, *n*, *o*, practically shut off, even at the base of the filaments (see Fig. 1).
4:40 P.M.	Stopped oxygen and began ventilating animal's lungs with expired air.
4:45 P.M.	Blood begins to flow through capillaries at tip of filament (evidence of capillary constriction at base of filament). Respiratory movements returning. Blood in fifth aortic arch becoming darker.
4:50 P.M.	Gill circulation opening up. Filaments seem to be larger and more fluffy. Pulse rate: 41 beats in 43 sec.
5:35 P.M.	Started oxygen through lungs, which are distended in a few seconds. Circulation in gill capillaries normal. Distension of lung with oxygen seems to dilate lung capillaries.
5:37 P.M.	Blood in aortic arches has become bright light red.
5:44 P.M.	Pulse rate: 62 beats per 60 sec. Filaments becoming pale.
5:45 P.M.	Blood still flowing through capillaries at tip of filament. Other capillaries in the filament have closed.
5:49 P.M.	Even the blood in the sixth aortic arch is light red.
6:00 P.M.	Capillaries in gill completely closed off. Blood flows through the arteriole loop.
6:10 P.M.	Same as foregoing. Heart rate: 62 beats per 60 sec.
6:20 P.M.	Condition the same.

In general, it was observed that the capillaries in the first gill closed first, then those in the second, and that the last ones to react were those in the third gill. This may be related to the action of the spiral valve which sends most of the blood from the lungs to the first gill, a small portion of it to the second gill, and very little to the last gill and sixth aortic arch.

As it had been established that a change from venous (reduced) to oxygenated blood caused a cessation of blood flow in the gill capillaries, and that this was not due to a slow pulse, the following question arose: Was the cessation of the flow of blood through a gill due entirely to the constriction of the gill capillaries, or could this be related in any way to the reactions of the anastomotic vessels at the base of the gill?

This question was answered by making direct observations on the anastomosis at the base of the gill during oxygen treatment. Before the blood was thus oxygenated in the lungs, no blood flowed through some of the anastomotic vessels *b* (see Fig. 1); they were, therefore, not noticed until 3 minutes after the oxygen treatment was started. These, as well as all other anastomotic arterioles, dilated gradually and finally carried a wide stream of blood. Eventually, most of the blood passed directly to the dorsal aorta through the anastomosis, and very little passed through the gill. Ten per cent carbon dioxide was then passed through the lungs. The blood in the aortic arches became darker, and the arterioles in the anastomosis gradually constricted. Blood soon stopped flowing in many of the arterioles of the anastomosis. Eventually, most of the blood passed through the gill proper, and only a relatively small quantity passed through the anastomosis.

The question also arose as to whether the reaction was due to a reflex related perhaps to distension of the lungs or stimulation of afferent fibers in the gills. That the reaction was not a reflex was evident from the speed of the reaction and the long latent period. The blood did not stop flowing in the branchial capillaries suddenly. On the contrary, only a few capillaries at a time closed off, and the whole reaction took place so gradually that it sometimes extended over a period of 20–30 minutes. Meanwhile, the blood in the aortic arches, judging from the color, became more and more saturated with oxygen. The reaction time was also related to the degree of ventilation of the lungs and to the condition of the circulation in the lungs. In the second place, the reaction cannot be a reflex caused by the distension of the lungs, because, if expired air, nitrogen, and nitrogen plus carbon dioxide were used to distend the lungs, just the

opposite effect was produced. Blood circulated through the gill capillaries at a tremendous rate.

In spite of all this evidence against the possibility of the reaction being due to a reflex, the following experiment was performed. An animal was prepared and cannulated in the usual manner. All the gill capillaries were closed off by passing oxygen through the lungs. Fifteen per cent carbon dioxide was then passed through the lungs to cause blood to circulate in the gill capillaries. Having thus established the fact that the animal reacted in a normal manner, all the nerves to the lungs and gills were cut on one side. This included sectioning the vagus and glossopharyngeal nerves close to the chondrocranium. The oxygen experiment was then repeated, and observations made on the gill vessels on both the operated and unoperated side. The reaction occurred on both sides at the same rate and degree.

In another animal the same procedure was followed, and the same results obtained. Then the other lung and gill were also denervated, and the lungs inflated with oxygen. The gill capillaries again closed and reopened when the lungs were ventilated with carbon dioxide. This is additional evidence that the reaction cannot be dependent upon a reflex. It was concluded from these experiments that changes in oxygen and carbon dioxide tension have a direct effect on the blood vessels in the gills.

DISCUSSION

The perfusion experiments show that adrenalin causes a great increase in the rate of flow through the gill proper peripheral to the anastomosis, while it causes a decrease in the rate of flow through the anastomosis. The decrease in the rate of flow through the anastomosis when perfused with adrenalin is not nearly as great as the increase in perfusion rate which adrenalin produces in the gill. It is not the magnitude of the reaction that is remarkable, but the fact that in the same gill arch we find some vessels which constrict, while others dilate, when perfused with adrenalin. If we should perfuse both sets of vessels at the same time, the reaction of one set would tend to negate the reaction of the other set. This explains why it was found impractical to perfuse all gills and gill arches at the same time.

It is interesting to note that while the reactions of the two sets of vessels are antagonistic, as far as the diameters of the vessels are concerned, they are synergistic with regard to the physiological effect on the entire animal. Any increase in the adrenalin content of the blood causes more blood to pass through the gill capillaries to be oxygenated, while less blood passes directly from the heart to the systemic vessels through the anastomosis at the base of the gill. The differential reaction produced by adrenalin would thus increase the efficiency of oxygenation of blood by the gills.

At the same time, the dilatation of the gill capillaries reduces the resistance to flow in the gill proper and would thus have a tendency to increase the blood pressure in the more peripheral systemic vessels. In this connection the results of Wyman and Lutz (1932) are interesting. These investigators found that the intravenous injection of adrenalin produced long-sustained pressor effects in *Squalus acanthias*. They measured the pressures in both the ventral and dorsal aortae. They found that the percentage increase of diastolic pressure in the dorsal aorta was consistently greater than that in the ventral aorta. They interpreted this as being due to a vasoconstrictor action of adrenalin peripheral to the gill capillaries, but admit that the region of action was not located. In my opinion, the fact that the percentage increase in the diastolic pressure in the dorsal aorta was consistently greater than that in the ventral aorta cannot be explained by a simple vasoconstrictor action of adrenalin peripheral to the gills. There must be in addition to this action a dilatation of the vessels in the gills. That adrenalin dilates the branchial vessels was shown by Krawkow (1913) in fishes and by Keys and Bateman (1932) in eels.

Probably the most interesting and fundamental fact that emerges from the present work is the observation that in the same organ, one set of vessels dilates while another constricts, when treated with the same substance. That histamine constricts the arterioles and dilates the capillaries was pointed out by Dale and Richards in 1918. The results of Dale and Richards are discussed because these investigators demonstrated a differential reaction of blood vessels to histamine and adrenalin in very low concentrations. Their results have been confirmed over and over again, but it must not be for-

gotten that the conclusion was the result not of direct observations on the capillaries but of physiological analysis. In the work presented in this paper, the differential vascular reaction to adrenalin was demonstrated by direct observation.

An increase in the carbon dioxide content of the blood going to the branchial arches also causes the anastomotic vessels to constrict and the gill capillaries to dilate. However, it might be added that, like adrenalin, it causes more blood to flow through the gill capillaries to be oxygenated, and less blood to flow directly from the heart to the dorsal aorta through the anastomotic vessels. This is, again, the result that one would expect.

The vessels of a branchial arch also react in a differential manner to changes in oxygen tension, but it should be emphasized that the reactions are just the reverse of those produced by adrenalin or carbon dioxide. Oxygenated blood causes the anastomotic arterioles to dilate and the gill capillaries to constrict.

This differential reaction of the branchial vessels, i.e., a dilatation of the anastomotic arterioles and a constriction of the branchial capillaries when they are supplied with oxygenated blood, is a truly remarkable mechanism. It prevents the waste of energy that would be necessary to force oxygenated blood through the gill. Instead of having to pass through the gill capillaries, the oxygenated blood is shunted directly to the systemic vessels through the anastomosis, *b* (see Fig. 1). It is this same mechanism that makes it possible to reduce the gills when, during a normal metamorphosis, more and more oxygen begins to enter the blood at other respiratory surfaces. This supports the hypothesis that the oxygenation of blood at points other than the gills is one of the factors responsible for gill reduction.

This recalls the attempts of Weisman (1875), of von Chauvin (1876, 1885), and of Boulenger (1913) to produce metamorphosis by enforced air breathing or by purely external non-glandular factors. Many of these attempts were doubtless successful, but later investigators have obtained varying and conflicting results (Powers, 1903; Huxley and Hogben, 1922; Huxley, 1925). One is given the impression, however, that there is something in the external environment (oxygen perhaps) that plays some rôle in the production of metamorphic changes. Recently (Figge, 1934), it was shown that it was

impossible to produce gill reduction in animals in which the sixth aortic arch was ligated, and which were kept in an environment of low air pressure (60 cm.).

The present paper explains how oxygen may influence gill reduction. Oxygenation of the blood in the lungs, skin, or pharynx as shown in this paper would cause the blood to stop flowing through the gill. In experiments that have not been published as yet, except in thesis form, it was shown that stopping the flow of blood through a gill by surgical means causes the gill filaments to be reduced. By stopping the flow of blood through a gill, the oxygenation of blood at points other than the gills is one of the factors responsible for gill reduction.

SUMMARY AND CONCLUSIONS

1. A technique was developed which permitted the perfusion of a single gill arch of *Amblystoma tigrinum*. This technique was later used to perfuse the separate parts of a gill arch. The gill proper was separated from the anastomosis at the base of the gill, and each part was perfused separately.

2. The addition of adrenalin to the perfusion fluid caused an increase of from 100 per cent to 200 per cent in the perfusion rate in isolated gills of *Amblystoma tigrinum* larvae (for anatomical diagram see Fig. 1; for result see graph, Fig. 2).

3. The addition of adrenalin to the perfusion fluid caused a decrease in the perfusion rate of the anastomosis at the base of the gill (for anatomical diagram see Fig. 1; for result see graph, Fig. 3).

4. By applying adrenalin to individual gills and parts of gills, and making direct observations on the branchial vessels of the intact animal, it was established that the capillaries in the gill filaments (*m*, *n*, *o* in anatomical diagram, Fig. 1) dilate tremendously under the influence of adrenalin.

5. This double reaction, i.e., constriction of the anastomotic arterioles at the base of the gill and dilatation of the gill capillaries of the same gill arch, which is caused by the same stimulus or substance, is called the "differential reaction" of the vessels of a branchial arch.

6. Ventilation of the lungs of *Amblystoma tigrinum* larvae with 10–15 per cent carbon dioxide induces differential reactions of the

vessels of a branchial arch which are the same as those produced by adrenalin. The anastomotic arterioles constrict, while the gill capillaries dilate. More blood therefore flows through the gill capillaries to be oxygenated.

7. Ventilation of the lungs with oxygen also induces differential reactions of the vessels of a branchial arch; but the reactions are just the reverse of those produced by carbon dioxide, i.e., oxygenation of the blood in the lungs causes the anastomosis at the base of the gill to dilate and the gill capillaries to constrict. The reactions of the two sets of vessels are antagonistic as far as the diameters are concerned but synergistic in regard to the physiological effect produced. As a result of these reactions, the oxygenated blood cannot flow through the constricted gill capillaries; it flows, instead, directly to the dorsal aorta and systemic vessels by way of the dilated anastomotic arterioles.

8. This work is presented as additional evidence in favor of the hypothesis that a change in the oxygen content of the blood sent to the gills is a factor in gill reduction.

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REGIONAL DIFFERENCES IN EYE-FORMING CAPACITY OF THE EARLY CHICK BLASTODERM AS STUDIED IN CHORIO-ALLANTOIC GRAFTS

(Four figures and one plate)

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THE present investigation is concerned with relative eye-forming capacities of various regions of the chick blastoderm in stages from late primitive streak through head process up to and including early somite stages. It is proposed (1) to determine the position, extent, and boundaries of the area with potencies for eye production in late streak and head-process stages, (2) to analyze the relative eye-forming capacities of the various regions within this area, and (3) to consider the evidence for a changing organization with respect to eye-forming potentialities at different stages in development.

There is present in the blastoderm of chicks of late streak or of head-process stages a definite region with eye-forming capacities. It is possible to determine the level in the embryonic axis and to define with a fair degree of accuracy the boundaries of the eye-forming area under the conditions of chorio-allantoic grafting.

In normal development the optic vesicles arise by lateral outpouchings of the walls of the forebrain. If we trace development backward to late streak and head-process stages, it is apparent that the prospective eye-forming areas are bilateral in position. In amphibians, Woerdemann (1929), Petersen (1924), and Manchot (1929) have shown by using vital staining technique that the eyes arise from bilaterally situated areas.

Two interpretations have been offered regarding the position and characteristics of the eye-forming areas. Spemann (1904, 1912) advanced the view from a study of *Triton*, *Bombinator*, and *Rana* that eye-forming areas are bilaterally situated and determined at the neurula stage, and that this determination extends even to specific

parts of the eye. Ekman (1914) and Fischel (1921) support Spemann's interpretation.

According to Stockard (1909, 1913), the eye area is first median and then by cell movements of eye-forming materials becomes bilateral in position. This view is based on experimental production of cyclopia in *Fundulus* and defect experiments on the neural plate of *Ambystoma*. Adelmann (1930) has shown for the urodeles that in the medullary plate stage median and lateral regions of the anterior end of the medullary plate have the capacity for eye production. These results favor the view of a single area with eye-forming potencies which includes lateral, as well as median, regions. The greatest capacity for eye production was demonstrated to lie in the median region.

The results of the present investigation demonstrate that in chick blastoderms of late streak and early head-process stages, prospective eye-forming potencies are present in median and lateral regions at the levels of the anterior part of the node and of the anterior end of the notochord, respectively. As in the urodeles, the capacity is greater in median than in lateral pieces.

A distinction must be made here between the prospective potencies and prospective significance of certain regions for eye production, since the areas showing eye-forming capacity may not coincide exactly with the areas which actually give rise to the eyes normally. The extent to which these coincide cannot be determined from the results of the present investigation. The experiments reported here deal with the potencies and are not directly concerned with the prospective value of the various areas.

Results obtained from experiments on late streak and head-process stages, namely, that median as well as lateral regions have eye-forming capacities, led to experiments in which an attempt was made to determine at what stage in development the median region loses this capacity. The results here are of interest in that up to the nine-somite stage narrow median strips from the brain floor still gave eye tissues under the conditions of the experiment.

This work represents a portion of a larger program undertaken by Dr. B. H. Willier and his students to map out the various organ germ areas of early chick blastoderms and to study their potentiali-

ties under the conditions of chorio-allantoic grafting. The advice and criticisms of Dr. Willier, who has supervised this investigation, have been responsible in large measure for the progress made. I should like to express my appreciation of his assistance. Also, I with to express my appreciation of the excellent facilities provided at the University of Chicago and the University of Rochester which enabled me to carry out this investigation.

MATERIAL AND METHODS

The method employed is essentially that described by Willier (1924). A chick blastoderm of desired stage in development is removed from the yolk and placed in a Syracuse watch glass. The embryo is kept in 0.9 per cent sodium chloride solution at a temperature of 39° C. throughout the operation. In a watch glass the blastoderm is flattened out, and by means of an ocular micrometer measurement of the length and width of the pellucid area and the lengths of the primitive streak and head process are recorded. These measurements, together with structural characteristics, are utilized in ascertaining the stage of the donor embryo.

Fine glass needles are employed in making the desired isolations. By placing the needle on the blastodisk and applying slight pressure, clean, accurate cuts are made with a minimum destruction of tissue. Each implant thus isolated is transferred in a drop of saline solution by means of a Spemann micropipette to the chorio-allantoic membrane of a host embryo previously incubated for 9 days. The grafted tissue becomes incorporated in the vascularized membrane of the host. After a period of 9 days, during which the graft grows and differentiates, it is recovered for histological examination. The grafts were fixed in Bouin's solution, sectioned at 8 μ, and stained with Heidenhain's iron haemotoxylin.

In connection with the study of the grafts preparations of 9-day normal embryos and a complete series of whole mounts of primitive streak, head-process, and early somite stages were examined. Some exceptionally fine slides of normal embryos, both serial sections and whole mounts, were loaned to me by Dr. B. H. Willier and Miss Mary E. Rawles, of the Zoölogy Department, University of Rochester.

DESCRIPTION OF DONORS AND ISOLATION OF IMPLANTS

The donors consisted of late streak, head-process, and early somite stages. The pellucid areas of late primitive streak stage used in the present experiments average 1.69 mm. in width, with a range from 1.26 to 2.18 mm., and average 2.67 mm. in length, with a range from 2.01 to 3.12 mm. The primitive streaks vary in length from 1.34 to 1.89 mm., with an average of 1.66 mm. The streaks in all instances are sufficiently far advanced to display definite primitive pits. The foregoing measurements are based on a total of 270 cases.

In the head-process stage the pellucid area varies in length from 2.52 to 3.57 mm., with an average of 3.02 mm.; in width the variation is from 1.47 to 2.31 mm., with an average of 1.80 mm. There is considerable variation in lengths of streak and head process, as this series includes all stages from the beginning process up to the first indication of a head fold. The range in length of head process is from 0.08 to 1.6 mm., with an average of 0.57 mm. The primitive streak ranges in length from 1.26 to 2.18 mm., with an average of 1.64 mm. Since the head process is gradually increasing and the streak correspondingly decreasing in length as a result of the backward movement of the node, the relative lengths of the two furnish a check on the developmental stage. The preceding measurements are based on a total of 372 cases.

The early somite stages used as donors comprise a continuous series of stages from presomite to twelve somites. This is the developmental period in which the morphogenesis of the neural tube is in progress. In the earliest stages (presomite to five somites) the neural folds are rolling up to form a tube. From the fifth to about the eighth somite stage a fusion of the neural folds occurs, and the walls of the diencephalon region are evaginating to form the optic vesicles. Foregut and heart are also in the process of formation during this period (Lillie, 1908, chap. v).

In late streak stages the primitive pit furnished a convenient center for describing the location of the various regions to be used as implants. The cuts are made at varying distances anterior, posterior, and lateral to the pit. A representative composite picture of the cuts made is shown in Figure 1. The distance of the cut from the pit is given in millimeters.

For purposes of analysis the letters "A," "Cb," "Ca," and "B" are assigned to the different levels of the blastoderm used in this study. It will be seen that there is considerable overlapping between the different levels. However, level A always refers to the region separated anterior to a transverse cut made in front of the pit. Posterior to this cut in each case is level Cb, which includes the pit level plus a level posterior to it. The anterior limit of the B level is at a variable distance posterior to the primitive pit. The region anterior to this level includes the pit level plus level A, and is consequently designated as level "Ca." The distance of the most posterior cut from the pit varies. An attempt was made, however, to make the areas of the levels involved in the isolation approximately equal.

Longitudinal cuts made at varying distances from the mid-line intersected the transverse cuts at right angles dividing the levels into corresponding left, median, and right pieces. The isolations are completed by making cuts around the anterior margin of the pellucid area.

Six pieces are thus isolated from each donor, as follows: left, median, and right from levels A and Cb or left, median, and right from levels Ca and B. In some instances, only the median and lateral pieces of one level are utilized.

Head-process stages are treated essentially the same as the streak stages, except that the anterior end of the notochord serves as the center about which the cuts are made. The designation of levels here is the same as in the case of streak stages (A, Ca, Cb, and B). Transverse and longitudinal cuts divide the area to be tested into left, median, and right pieces at these levels. The most posterior cut is made anterior to Hensen's node, except in short head-process stages, where it is necessary to include the node in the posterior level to maintain approximately equal areas in the levels (Fig. 2).

A third series of experiments, suggested by the results obtained from streak and head-process stages, was to isolate a very narrow strip from the median region of still older embryos to test its capacity for eye production. A piece 0.084 mm. average width (range from 0.06 to 0.1 mm.) by 0.4–0.5 mm. long was taken from the prospective brain-floor region of donors ranging from presomite to the twelve-somite stage. The notochord is always included in the piece taken. In

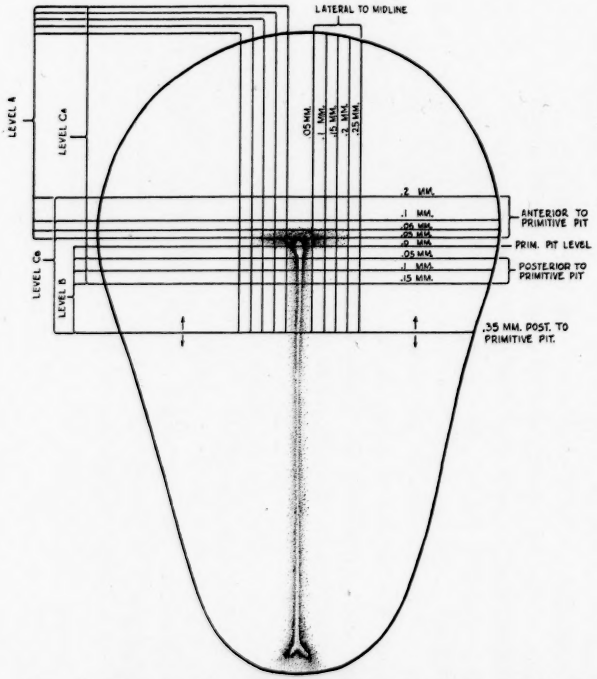


FIG. 1.—Diagrammatic representation of various cuts made in the isolation of implants at late streak stages. The position and extent of the eye area is shown. The density of stippling indicates roughly the frequency with which eye parts differentiate within the area.

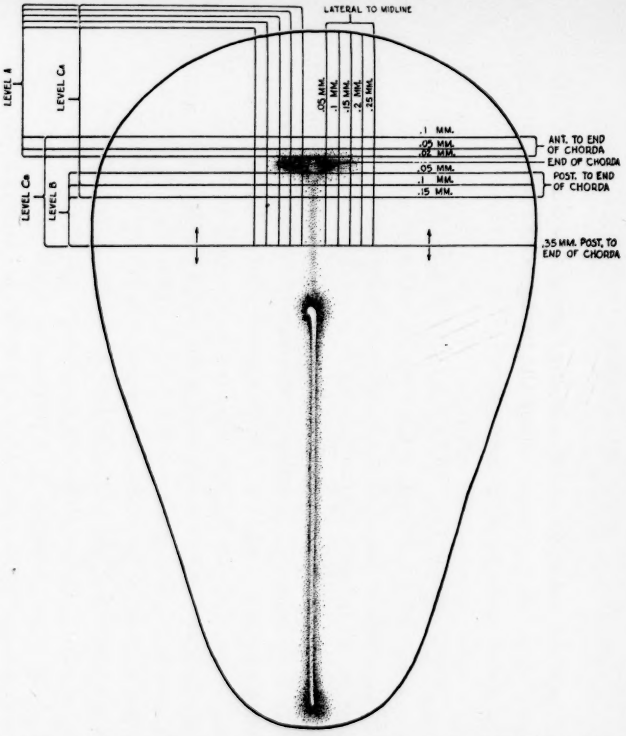


FIG. 2.—Diagrammatic representation of various cuts made in the isolation of implants at head-process stages. The position and extent of the eye area is shown. The density of stippling indicates the eye-forming frequency of various parts of the area.

some cases the head fold and developing foregut is included with the implant. After the five-somite stage the fusing neural folds must be spread apart before making the isolation.

EXPERIMENTS CONCERNING EYE-FORMING CAPACITY

I. POSITION, EXTENT, AND BOUNDARY OF THE EYE AREA

At late primitive streak stages in development.—From an examination of grafts developed from implants of various regions of late streak stages, it is determined that an area extending from the primitive pit laterally, anteriorly, and possibly posteriorly has the capacity for eye production. Figure 1 shows the eye-forming area of a late streak stage as determined by an examination of a total of 107 grafts yielding 35 cases in which eye parts differentiate.

From a total of 15 grafts obtained from implants more than 0.16 mm. lateral to the primitive pit, one shows differentiation of eye. This case resulted from a left implant of the primitive pit level isolated by a cut 0.18 mm. from the mid-line. Eight of the 13 grafts in this group were obtained from pieces 0.2–0.24 mm. from the pit. None of these 8 grafts contained eye material. It seems probable from these results that about 0.18 mm. represents the approximate lateral extent of the eye area, although the number of grafts included in the analysis is too small to establish this point.

The anterior and posterior limits are much more sharply defined than the lateral, which probably means that the level in the host axis, from which eye can differentiate, is quite definitely fixed even at this early stage in development. In 12 grafts from regions more than 0.06 mm. anterior to the primitive pit no cases of eye were recorded. Of a total of 24 grafts from 0.03 to 0.06 mm. anterior, 8, or 33 per cent, produced eye tissue. From the primitive pit to the anterior edge of the node is about 0.06 mm. Thus all these anterior regions which gave eye included a small amount of the node material.

Posterior to the pit the frequency of eye production is extremely low. Only one case has been obtained. This came from a left piece taken posterior to a cut made 0.02 mm. behind the pit level. Hunt (1932) reports no cases of eye in 25 grafts from implants taken posterior to a transverse cut through the pit.

Summarizing it may be observed that at the late primitive streak stage, any part of an ellipsoidal area extending from the primitive pit approximately 0.06 mm. anterior, 0.18 mm. lateral, and possibly 0.02 mm. posterior has the capacity to produce eye parts.

At head-process stages in development.—Figure 2 is a diagrammatic representation of the eye area as it relates to other regions of the chick blastoderm at the head-process stage in development. The position and extent of the area figured was determined by the examination of a total of 131 grafts, of which 56 contained eye tissue.

The area which has eye-forming capacities is located at the level of the anterior end of the notochord. Although this region has been demonstrated to have such a capacity by Hunt (1931), Rudnick (1932), and Stein (1933), its exact position, extent, and boundaries have not been determined previously.

An analysis of the data shows that the center of this area lies at or near the anterior end of the notochord and extends out from this center in all directions. To ascertain the extent of this area, cuts are varied in an antero-posterior and also in a mediolateral direction as described above. Measurements are made of the distance of each cut from the anterior end of the notochord. This variation is from 0.02 to 0.1 mm. anterior, from 0.04 to 0.2 mm. posterior, and from 0.05 to 0.24 mm. lateral to the anterior end of the notochord.

In the posterior direction within 0.1 mm. from the anterior end of the notochord 5 cases of eye were obtained from a total of 20 grafts, for a percentage of 25. Of these 5 cases, 1 was from left, 3 from median, and 1 from right regions. Beyond 0.1 mm. no eye tissue was present in 11 grafts.

The anterior limit is apparently closer than this to the anterior end of the notochord. From 13 grafts within 0.05 mm. anterior to the notochord 7 cases of eye were obtained. Here also left, median, and right regions are represented. Beyond 0.05 mm., however, no eye tissue was obtained from 5 grafts. Incidentally, it should be noted that graft frequency is low at this extreme anterior level.[1]

In a mediolateral direction the extent of the area is not so well defined; but of a total of 11 grafts from lateral pieces, 0.16 mm. or

[1] I am indebted to Dr. B. H. Willier and Miss Mary E. Rawles for 6 grafts included in the tabulations of level A.

more from the mid-line, only 1 case of eye was obtained. This came from a left piece beyond 0.19 mm. lateral to the mid-line. Six of the grafts were from pieces more than 0.2 mm. from the middle. This case of eye was obtained from the C level (level of the anterior end of the notochord), which may mean that the lateral extent of the area is greatest at this level.

Summarizing, it is observed that eye is obtained from regions as far as 0.05 mm. anterior and 0.1 mm. posterior to the anterior end of the notochord and as far lateral as 0.19 mm. from the mid-line. From these data a map of the area with eye-forming capacity has been made (Fig. 2). The area is somewhat ellipsoidal in outline; its transverse diameter is about 0.38 mm., and its longitudinal diameter is 0.15 mm.

The number of grafts examined is too small to permit the statement that this is the absolute limit of the area with the capacity to produce eye. Such a statement could be based only on negative results in large numbers of grafts from regions outside the area outlined. However, it seems reasonable to conclude that the area defined above includes by far the larger portion of the region with eye-forming potencies, as expressed in chorio-allantoic grafts.

The limitations of the method make it impossible to determine whether a narrow strip of cells in the median line lacks the capacity to produce eye. However, grafts obtained with both pigmented and sensory layers of the retina from median strips less than 0.08 mm. wide or one-twentieth the width of the pellucid area makes it seem highly improbable that a strip lacking this capacity is present.

A comparison of the areas with eye-forming capacity in late streak and head-process stages shows that they are similar as to size, shape, and extent. However, the area is located at the primitive pit in the late streak stage and at the anterior end of the notochord in the head-process stage. When we consider the manner of formation of the notochord (i. e., by the backward movement of the primitive knot, leaving a strand of cells behind), it becomes apparent that the two positions are entirely comparable as to level in the axis. The medio-lateral extent of the area is practically the same for both stages. However, the antero-posterior extent is much greater in the

head process than in the late streak stage. This may be accounted for by the fact that during the formation of the head process there is an elongation of the blastodisk in an antero-posterior direction. The evidence presented above would seem to show then that the two areas are comparable as to position, extent, and boundaries.

II. FREQUENCY DIFFERENCES IN EYE-FORMING CAPACITY WITHIN THE EYE AREA

The eye differentiates in grafts from both median and lateral regions at the level of the anterior end of the notochord at head-process stages and the level of the node in late streak stages. The median regions have a greater capacity for eye production than do the lateral pieces. This is revealed in these grafts in two ways: first by a study of the relative eye frequencies, and second by an analysis of the morphogenesis and histogenesis of the eye.

At late primitive streak stage.—Table I summarizes the data on the relative frequencies of the various regions within the eye-forming area. From median pieces of levels A, C, and B (Fig. 1) 48 per cent, or 19 of the 40 grafts studied, contained eye material. Left pieces from the same levels produced eye material in 27 per cent, or 10 of the 37 grafts recovered; while 6 grafts (20 per cent) of the 30 obtained from comparable right regions showed differentiation of eye tissue. From the C level alone the eye-frequency is higher in both median and laterals, i. e., median 81 per cent, left 44 per cent, and right 43 per cent. However, the relation between medians and laterals remains about the same as for all levels taken together.

From this analysis, two important differences in eye-forming capacity are observed: first, the median is higher than either of the laterals; and second, the left is higher than the right. Not only is there a difference in eye-forming frequency, but a corresponding difference in frequency of grafted piece to survive. The median region is highest (45 per cent), left next (40 per cent), and right lowest (34 per cent).

Carrying the analysis still farther, it may be seen that ability of grafts to survive, as well as frequency of eye-differentiation from lateral pieces, tends to decrease as the distance of the lateral piece from the mid-line increases (Table I).

A comparison of frequency of eye differentiation in level C (the level of the primitive pit) and levels anterior and posterior to it shows a decrease in eye-forming capacity in both directions. The decline is much more rapid per given distance, anteriorly and posteriorly from the pit, than has been observed in a medio-lateral direction. This is shown in Table I, where it may be noted that from 0.03 to 0.06 mm. anterior to the pit the frequency of eye formation is 33 per cent, as compared with 57 per cent for the pit level. Posterior to the

TABLE I

EYE-FREQUENCIES FROM MEDIAN AND LATERAL PIECES AT VARIOUS TRANSVERSE LEVELS (LATE PRIMITIVE STREAK STAGE)

DISTANCE OF CUT ANTERIOR AND POSTERIOR TO PRIMITIVE PIT		LEVEL A ANTERIOR TO PRIMITIVE PIT						LEVEL C PRIMITIVE PIT			LEVEL B POSTERIOR TO PRIMITIVE PIT						TOTALS			PERCENT		AVERAGE PERCENT	
		.07-.2 MM.			.03-.06 MM.			.00 MM.			.00-.05 MM.			.06-.16 MM.									
	DISTANCE OF CUT FROM MIDLINE	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	GRAFTS	EYES	GRAFTS	EYES
LEFT	.16-.24 MM.	3	2	0	2	1	0	13	5	1	2	0	0	9	1	0	29	9	1	31	11		
	.11-.15 MM.	6	2	0	11	4	2	13	8	3	7	4	0	4	2	0	41	20	5	49	25	40	27
	.05-.10 MM.	4	0	0	0	0	0	9	3	3	5	3	1	5	2	0	23	8	4	35	50		
MEDIAN	.16-.24 MM.	2	1	0	2	1	1	6	3	2	0	0	0	4	1	0	14	6	3	43	50		
	.11-.15 MM.	7	3	0	17	8	4	15	9	7	7	2	0	2	1	0	48	23	11	48	48	45	48
	.05-.10 MM.	4	2	0	3	3	1	10	4	4	3	1	0	6	1	0	26	11	5	42	45		
RIGHT	.05-.10 MM.	3	1	0	0	0	0	12	6	3	4	1	0	3	2	0	22	10	3	45	30		
	.11-.16 MM.	6	1	0	15	6	0	12	5	3	6	0	0	4	2	0	43	14	3	33	21	34	20
	.16-.24 MM.	2	0	0	1	1	0	10	3	0	2	0	0	9	2	0	24	6	0	25	00		
TOTALS		37	12	0	51	24	8	100	46	26	36	11	1	46	14	0	270	107	35				
PERCENT GRAFTS		32			47			46			31			30			4						
PERCENT EYES		00			33			57			9			00			33						

pit the drop is even more abrupt; i.e., to 9 per cent within 0.05 mm. of the pit. In fact, no eye material was obtained from grafts beyond 0.02 mm. posterior to it.

At head-process stage.—As described above, cuts made on each side of the mid-line at distances varying from 0.05 to 0.24 mm. isolated left, median, and right regions. By varying the cuts in this manner, it is possible to see what effect the inclusion or exclusion of a certain region has on the presence of certain structures and also on the differentiating capacities of the various areas. This method has been utilized here to test the difference in eye-forming capacity of various regions.

A tabulation of the case numbers from left, median, and right regions of levels A, C, and B according to the distance of the cuts from the anterior end of the notochord is contained in Table II. The frequencies are given in percentages. An analysis of these results shows that eye frequency is highest in the median (52 per cent), next in left (43 per cent), and lowest in right (27 per cent). There is a gradual increase in eye frequency of lateral pieces as the region includes more of the median area. On the left this increase is from 20 to 48

TABLE II

EYE-FREQUENCIES FROM MEDIAN AND LATERAL PIECES AT VARIOUS TRANSVERSE LEVELS (HEAD PROCESS STAGE)

		LEVEL A ANTERIOR TO NOTOCHORD						LEVEL C ANTERIOR END OF NOTOCHORD			LEVEL B POSTERIOR TO ANTERIOR END OF NOTOCHORD						TOTALS			PERCENT		AVERAGE PERCENT	
DISTANCE OF CUT FROM ANTERIOR END OF NOTOCHORD		.06-1 MM.			.02-.05 MM.			.00 MM.			.04-.1 MM.			.1-.2 MM.									
	DISTANCE OF CUT FROM MIDLINE	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	HOSTS ALIVE	NUMBER OF GRAFTS	NUMBER OF EYES	GRAFTS	EYES	GRAFTS	EYES
LEFT	.16-.24 MM.	5	1	0	7	0	0	15	3	1	1	1	0	1	0	0	29	5	1	17	20		
	.11-.15 MM.	11	1	0	5	1	0	38	11	7	1	1	0	5	2	0	60	16	7	27	44	33	43
	.05-.10 MM.	0	0	0	3	1	1	26	15	9	8	4	1	7	3	0	44	23	11	52	48		
MEDIAN	.16-.24 MM.	4	1	0	5	2	2	17	10	7	2	1	0	0	0	0	28	14	9	50	64		
	.11-.15 MM.	9	1	0	6	1	1	32	14	10	2	1	0	5	1	0	54	18	11	33	61	42	52
	.05-.10 MM.	0	0	0	3	2	1	23	11	4	12	6	3	8	3	0	46	22	8	48	36		
RIGHT	.05-.10 MM.	0	0	0	5	3	2	20	5	3	6	3	0	6	1	0	37	12	5	32	42		
	.11-.15 MM.	9	1	0	6	1	0	29	10	3	2	2	1	3	1	0	49	15	4	31	27	30	27
	.16-.24 MM.	6	0	0	4	2	0	14	3	0	1	1	0	0	0	0	25	6	0	24	00		
TOTALS		44	5	0	44	13	7	214	82	44	35	20	5	35	11	0	372	131	56				
PERCENT GRAFTS		11			30			38			57			31			36						
PERCENT EYES		00			54			54			25			00			43						

per cent, and on the right from 0.0 to 42 per cent. As the size of the lateral piece increases, the corresponding median decreases. This results in a corresponding decrease in frequency in the median pieces. The difference in frequency may be due to the small size of the narrowest median piece grafted.

Parallel with the differences in eye frequencies are differences in graft frequencies, particularly between median and laterals; i.e., median pieces produce grafts in 42 per cent of cases, left in 33, and right in 30. That this is not merely a chance occurrence is emphasized by corresponding differences observed in similar transplants

of late streak stages. Apparently, medio-lateral differences exist in the capacity to produce graft, as well as in capacity to differentiate eye parts.

An analysis of the differences in eye-forming capacity of different levels emphasizes the importance of level in determining potentialities for organ formation. A comparison of eye-forming frequency of grafts from the level of the anterior and of the notochord (C level) and levels anterior (A level) and posterior (B level) to it shows that there is a marked decrease in both directions from the level of the anterior end of the notochord. Eighty-two grafts from level C gave eye material in 44 cases (54 per cent), as compared with 18 grafts yielding 7 cases of eye from level A and 20 grafts from level B, 5 of which contain eye. These figures include grafts from left, median, and right pieces isolated by cuts less than 0.1 mm. either anterior or posterior to the anterior end of the notochord.

These results, when combined with those described above for medio-lateral differences, show a grading-off in all directions from a region of relatively high eye-forming capacity to regions which entirely lack this capacity (Table II). The narrow antero-posterior extent of the eye area, plus the fact that the limits in both directions are quite well defined, indicates that the level for eye production has been established before this stage in development.

Figure 3 is a diagrammatic representation of these differences in terms of the distance from the mid-line of the cuts which separated the respective left, median, and right regions. The data used in constructing these graphs are summarized in Tables I and II. Although the curves do not show a smooth grading-off from the mid-line laterally, the tendency in each case is clearly indicated. The drop in frequency of formation of eye tissue from right regions is much more abrupt than from left regions, although a definite decrease is evident in both directions. The percentage values for corresponding regions in the two stages do not agree in all instances; but a definite similarity in the general trend of the two curves is evident; i.e., highest in the median, intermediate in height on the left, and lowest on the right.

Curves of the relative frequencies of the different levels from streak, as well as head-process, stages are shown in Figure 4. The

sharp decrease in each case is probably indicative of the importance of level in the host axis in determining the structures which differentiate. The difference in the sharpness of the decline in the two

FIG. 3

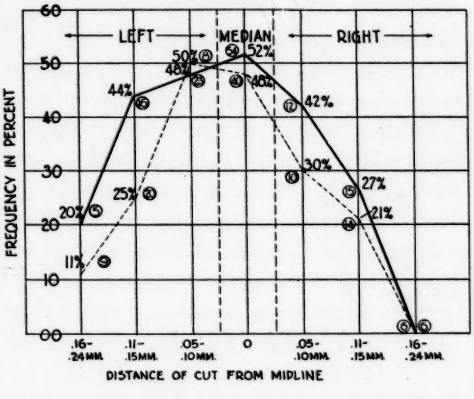


FIG. 4

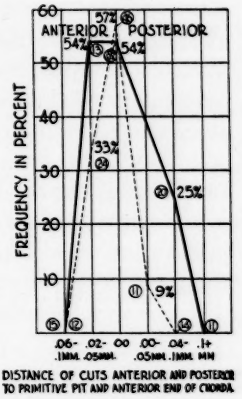


FIG. 3.—Graphic representation of the medio-lateral gradient in eye-forming frequency at late streak and head-process stages.

FIG. 4.—Graph showing the relative eye-forming frequencies of levels anterior and posterior to the primitive pit in late streak stages and the anterior end of chorda in head-process stages. (Late streak stages are represented by broken lines, and head-process stages by unbroken lines. The encircled figure is the number of grafts examined.)

cases may be accounted for partially at least by the fact that the blastoderm elongates considerably in the head-process stage, whereas the width is not increased proportionately.

III. EYE-FORMING CAPACITY IN EARLY SOMITE STAGES

The percentage of eye frequency for median regions at late streak stages (Table I) is 48, as compared with 52 at head-process stages (Table II), an increase of 4 per cent; while for left regions an increase of 17 per cent (from 27 to 43) and for right regions an increase of 7 per cent (from 20 to 27) is observed. A graphic representation of this relative difference between medians and laterals at these two developmental periods is contained in Figure 3. This figure is evidence of a gradual increase in eye-forming capacity in lateral regions. Both median and lateral regions show greater eye-forming capacity

in the later stages, but the rate of change is more rapid in the laterals than in the medians.

This line of evidence led to an analysis of the eye-forming capacity in younger and older stages than those just analyzed. The question as to how eye-forming potentialities of median and laterals compare in stages younger than those described above remains for future investigation. An attempt has been made, however, in the present investigation to analyze the condition in embryos still older than those considered above. For this purpose donors varying in age from presomite[2] to twelve somites were used. A piece of the brain floor was taken out at the level of the forebrain, and usually extending posterior to it. Pieces grafted were from 0.06 to 0.10 mm. in width by 0.4–0.5 mm. in length. They include in each case the underlying notochord. In some cases the head fold and foregut material was removed, and in others it was transplanted with the brain-floor material. What influence, if any, the removal of foregut has on the differentiation of eye is not revealed by the present experiments. Eye differentiated in grafts from pieces without foregut material.

TABLE III

EYE-FREQUENCIES FROM BRAIN FLOOR GRAFTS

STAGE	HOSTS ALIVE	No. OF GRAFTS	No. OF EYES	PERCENT GRAFTS	PERCENT EYES
1-3 SOMITE	17	6	4	35	67
4-8 SOMITE	17	7	5	41	71
9-12 SOMITE	9	4	0	44	00
TOTALS & AVERAGES	43	17	9	40	53

IMPLANTS RANGED IN WIDTHS FROM .063-.10MM

It is of interest to note that all of the grafts resulting from this extremely small implant are very small, but the differentiation is not noticeably different histologically from that in much larger grafts.

For convenience in making comparisons the grafts are divided into three groups according to the stage of the donor from which they came. Table III summarizes concisely the results obtained from each of these groups. The data show in the first place that eye is produced with a relatively high frequency in stages from presomite to eight somites. The percentages are approximately the same for the presomite to three-somite and the four-to-eight-somite groups. Secondly, it will be observed that from nine-to-twelve-somite stages

[2] "Presomite" is used here to refer to the stage in which the neural folds are beginning to arise but before the first somite is definitely indicated.

no cases of eye were obtained from 4 grafts. This result indicates that the eye-forming capacity, which is present in the brain-floor region from presomite to eight-somite stages, is lost during the following stages. A great many more grafts would be necessary to establish this point, but the results are of sufficient interest to justify their inclusion in this paper. It has been demonstrated, at least, that the capacity for eye production remains in this median region, even up to the stage at which the optic vesicles are beginning to form. A definite isolation of the potencies into the lateral primordia does not precede the beginning of the morphogenesis of the optic vesicles.

It is interesting to note here that in a series of 6 grafts of the lateral region, which is prospective eye material, eye tissue was present in every graft. When this result is compared with that for head-process stages, it becomes evident that a definite rise in the eye-forming potentiality of the lateral regions has occurred during this developmental period.

IV. QUALITATIVE DIFFERENCES WITHIN THE EYE AREA AS SHOWN BY A STUDY OF MORPHOGENESIS AND HISTOGENESIS

Regional differences in capacity to produce eye is very evident in the grafts studied in the present investigation. It is not my purpose in the present paper to consider in detail the qualitative differences in the various regions, but to describe more or less typical cases and to summarize the differences in capacity for differentiation of the regions under consideration.

Eye tissues differentiating in grafts do not display normal relationships, probably because of mechanical factors operating during morphogenesis. The histology, however, is essentially like that of normal tissues. This observation is substantiated by comparing the results of Weysse and Burgess (1906) on the normal histogenesis of the retina and of Hoadley (1925) on the histology of eye tissues in grafts.

Analysis of regional differences in eye-differentiating capacity in grafts from both head-process and late streak stages shows that the median region has a much greater capacity to form essential eye tissues consisting of sensory and pigmented layers of the retina and lens than do the lateral regions. However, in grafts from median regions all degrees of differentiation are present from a small amount of

PLATE I

FIG. 5.—A portion of an optic cup showing the various layers of the differentiated retina. The implant was a median piece of donor at head-process stage (see text for details). ×235.

FIG. 6.—An optic cup showing both pigmented and sensory layers of the retina; differentiated from a median piece of donor at late streak stage (see text for details). ×210.

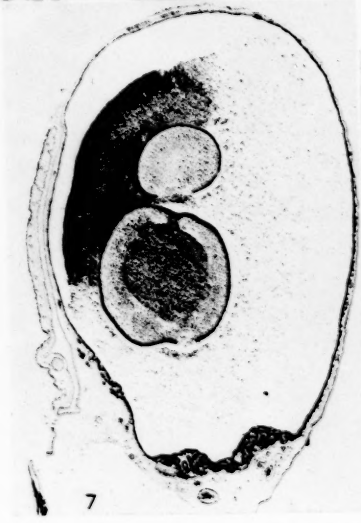
FIG. 7.—A large optic vesicle showing lens in contact with convoluted pigmented layer; differentiated from a left anterior piece of donor at head-process stage (see text for details). ×38.

FIG. 8.—A portion of a graft showing convoluted pigmented layer connected with forebrain material; differentiated from a right anterior piece of donor at head-process stage (see text for details). ×140.

PLATE I

5

6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8

pigmented layer to a definite optic cup with the differentiated retina. In Figure 5, a section of such an eye cup is shown. The donor from which the grafted piece was taken was in the head-process stage. The implant included the anterior 0.1 mm. of the notochord, 0.1 mm. on each side of the mid-line and extending anteriorly to the margin of the pellucid area.

Out of a total of 28 cases of eye tissue recorded from median pieces, 6 showed a differentiation comparable to the above mentioned case. In several other cases a much thickened layer, presumably retina, was found connected with the pigmented layer.

Figure 6 is a photomicrograph of a relatively normal-appearing optic cup. The donor in this case was at a late streak stage. The piece used as the implant was taken 0.06 mm. anterior to the primitive pit and included 0.10 mm. on one side and 0.12 mm. on the other side of the mid-line, as determined by the position of the pit. The piece extended anteriorly as far as the margin of the pellucid area.

Differentiation has not progressed as far as in the preceding case described, but the sensory layer is cupped back into a pigmented layer. In no instance was differentiated sensory layer obtained from late streak stages. This is probably a chance occurrence, as complete differentiation has been obtained from stages much younger than these (Butler, 1935).

The best cases of lens differentiation have been obtained from median regions. Three such cases have been positively identified. It is interesting to note that the ectoderm included with the implants in these cases does not normally contribute to the lens. In each of the three cases the lens is connected to skin in the graft. One possible explanation of the occurrence of lens in these grafts would be induction by the eye differentiated in the graft. In this connection it is also interesting to note that the eye tissue is derived from cells which normally do not contribute to eye formation.

When we proceed now to a consideration of the type of differentiation obtained from lateral pieces, it may be observed that no case of differentiated sensory layer has been recorded from any lateral region. However, definite vesicles are common from left lateral regions. Figure 7 shows a large vesicle entirely surrounded by pigmented layer, with a small lens shown at one point in contact with

the pigmented layer. This lens is continuous with definite skin in another section. Also in another section the pigmented layer is continuous with a cupped sensory tissue which is connected with the forebrain material seen in the center of the vesicle.

Figure 8 is quite typical of the differentiation found in right regions. Pigmented layer is present in a much convoluted form with no definite organization into an eye. It seems significant that in no case was a vesicle of the type quite common to grafts from left and median regions found.

It is also interesting to note that the quantity of eye material is smaller from right pieces than from other regions tested, and that in no case has lens been identified in grafts from right pieces but that three cases have been noted from left. The eye material is shown connected to a forebrain vesicle in the figure. This connection is typical in grafts obtained from left and median, as well as right, regions. The presence of eye in connection with nervous tissue is used as a criterion for identifying forebrain (Willier and Rawles, 1931).

By the foregoing analysis of the differentiation of eye tissue in grafts from median, left, and right regions, additional evidence is furnished for some fundamental difference in these three regions. The median region produced the highest frequency of both eye tissue and grafts and the best differentiation of eye in the graft. Furthermore, the percentage of graft survival, as well as eye formation and the type of differentiation obtained, was higher from left than from corresponding right regions. The fact that all three lines of evidence point in the same direction indicates that we are dealing with a general difference in activity of the three regions. This may be expressed as a medio-lateral gradient with a more rapid decline to the right than to the left.

DISCUSSION

The analysis of results has brought forth three generalizations with respect to eye-forming capacities of early chick blastoderms. First, in late streak and head-process stages an area is present more or less definitely localized and limited, any part of which has a capacity to produce eye tissue. Second, within this area there exists a medio-lateral, as well as an asymmetrical, difference in eye-forming capacity. Third, during the normal developmental processes there is

a progressive increase in the eye-forming capacities of lateral regions (the regions which normally produce eye). The median region (prospective brain-floor material) does not lose this capacity until after the eight-somite stage.

I. THE NATURE OF THE AREA WITH EYE-FORMING CAPACITY

It has been determined that the entire blastoderm at head-process stages in development is composed of organ-forming germ areas Rawles and Willier, 1934; (Willier and Rawles, 1935; and Rawles, 1935). The potential eye area then is only one of a great many such areas which overlap each other.

It has been shown previously that eye-forming capacity is present in different regions of the blastoderm at late streak stages (Hunt, 1932; Stein, 1933) and at head-process stages (Hunt, 1931; Rudnick, 1932; Stein, 1933). The present investigation has attempted to localize more definitely and to limit these regions.

The eye-forming germ area occupies a somewhat comparable position in both late streak and head-process stages. At late streak stages the primitive pit and at head-process stages the anterior end of the notochord are centers about which materials have eye-forming capacity. In both, the region with eye-forming capacity is ellipsoidal in outline with the greater diameter at right angles to the longitudinal axis of the embryo. The longitudinal diameter at late streak stages is much shorter than it is at head-process stages. Cells within these ellipsoidal areas have eye-forming capacities. In addition, these same cells may have the potentiality for various other ectodermal structures characteristic of the given level or region in which they are located. The determination process up to this time, then, is probably concerned only with restricting the area with potentialities for eye production, and not specifically and irrevocably determining certain cells for a specific organ. Potential forebrain material occupies approximately this same region and is found associated with eye in grafts from implants of any part of this area. Apparently, any part of the region, then, has the capacity to produce more than it does produce normally.

Since both sensory and pigmented layers of the retina and lens can be produced from various parts of this region, it is likely that the area is an equipotential system for eye production; i. e., any part is

capable of producing a complete organ, though very much reduced in size. Spemann and Bautzmann (1927) and Holtfreter (1931) point out that the presumptive medullary plate is equipotential. Adelmann (1929*a*, 1929*b*, 1930) has shown that in the urodeles the anterior end of the medullary plate is an equipotential system. In his experiments median or lateral regions of the anterior medullary plate were removed, and the remaining parts developed eyes. Also, these same areas transplanted separately to the belly wall produced eyes. By studying the eye-forming frequency of median and lateral pieces, Adelmann found that median regions have a much greater capacity for eye production than do laterals.

On the basis of what has been said, we cannot consider the eye area in chick embryos of these stages as either localized distinctly median (Stockard, 1913) or as bilateral primordia specifically determined (Spemann, 1912), but as an area occupying both median and lateral regions of the prospective neural plate material at the level of the primitive pit or the anterior end of the head process. The prospective eye regions in the chick are probably bilateral, as Woerdemann (1929), Petersen (1924), and Manchot (1929) have demonstrated for amphibians; but this certainly does not refer to potential singleness or doubleness of the eye anlage.

II. MEDIO-LATERAL AND ASYMMETRICAL DIFFERENCES IN EYE-FORMING CAPACITY

It has been pointed out that the median regions produce both grafts and eyes with greater frequency than do the laterals. Also, the type of differentiation of eye in grafts from median regions is better than from the laterals, as based on the quantity of eye material, the degree to which differentiation has progressed, and the relative normality of the eyes. It has been noted that a corresponding difference is present between left and right regions; the left is superior to the right in graft and eye production, as well as in type of differentiation.

The fact that the variation is in the same direction; i. e., best in median, next in left, and poorest in right, with respect to all three of these conditions in both late streak and head-process stages furnished additional evidence that some one generalized condition is responsible for all three results.

Graft frequency can be related to a difference in the capacity of the implants to become incorporated in the chorio-allantoic membrane or to differentiate once they become incorporated. Assuming a more or less constant medium of differentiation, the difference in the capacity of the various regions to produce grafts is a function of the activity of the cells implanted. Similarly, the capacity a graft has to produce all organs for which it has potentialities or to produce a particular organ every time depends on the general ability of the cells to express all their potentialities. This would determine the frequency with which any organ would occur in grafts.

Hyman (1927) has shown by susceptibility experiments that the node region in late streak and the corresponding region of the anterior end of the head process in head-process stages are the regions of greatest metabolic activity. The results of the present experiments bear this out, using the capacity for eye production as a measure of this difference. In addition, the present experiments indicate strongly that the left side is more active than the right; but on each side there is a gradual decrease in activity in a medio-lateral direction. Using eye frequency as a measure, there is a medio-lateral gradient in cell activity, with the decline more rapid to the right than to the left.

On the basis of the discussion just presented, Figure 3, showing graphically the relative eye frequencies of left, median, and right regions, may be offered as an indication at least of the relative differences in activity of the three regions.

III. EVIDENCE OF A CHANGING ORGANIZATION

In the preceding discussion an analysis has been made of the differences which exist in different parts of the eye area at head-process and late streak stages in development. This leads next to a consideration of the differences which exist between successive stages in the developmental process.

The data presented in the preceding sections give evidence of a regular and orderly change in the eye-forming capacities as development proceeds. An increase in the eye-forming capacity of lateral regions at head-process stages over lateral regions at late streak stages is indicated by comparison of eye frequencies. Also, the in-

crease observed in median regions from late streak to the head-process stage is not nearly as great as the lateral increase (cf. Tables I and II). When head-process stages were compared with still later stages; i. e., early somite stages, it was found that the capacity of the lateral regions (potential optic vesicles) had increased to 100 per cent, whereas the corresponding medians (potential brain floor) did not increase to more than 70 per cent. In stages beyond eight somites no eye tissue was present in 4 grafts from the brain floor.

These results are interpreted as indicating a shift in developmental potency for eye production from the median to the lateral regions. This process probably continues until the median region no longer has any eye-forming capacity, while the lateral regions produce eye every time the transplanted tissue develops into a graft. This is probably the stage in which the prospective eye areas attain specific potencies (Lillie, 1929). From this stage on, eye tissue alone would be produced by these areas, according to this interpretation.

During a comparable stage in urodeles prechordal mesoderm is shifting laterally in position, and mandibular arch material is being added from the neural crest (Adelmann, 1934). Cyclopia accompanies the failure of the prechordal mesoderm to shift laterally. Probably the activity of the mesoderm material brings about an increase in the activity of the lateral regions to an optimum for eye production, and consequently the greatest eye-forming capacity becomes located in the bilateral optic vesicles.

This explanation is supported by certain experiments in producing eye abnormalities in *Planaria dorotocephala* (Child, 1916, 1920, 1921, and 1924, pp. 107–10). By subjecting the animals to inhibiting agents during regeneration, it was found that the medio-lateral gradient could be inhibited to varying degrees so that the median region with the highest metabolic rates would be reduced and the bilateral organs approximated to the median line. In other words, the median region now assumes the metabolic rate characteristic of the bilateral organs (in this case eye), so that the eyes differentiate at or near the mid-line. If this interpretation is correct, there is a shift of eye-forming potentialities toward the mid-line as a result of an inhibition of the medio-lateral gradient; whereas in normal development the potentially single eye-forming area becomes divided into

bilateral primordia as a result of a stimulating influence exerted on the ectoderm, probably by increased mesodermal activity.

Wright and Wagner (1934), in accounting for head abnormalities in guinea pigs, picture the existence of various centers of activity at certain critical stages in development. The optic field, which is single and median, is one of these centers. If inhibitions, either genetic or environmental, are supplied to this center at the critical period, the physiological processes by which this field produces bilateral eyes is interrupted, resulting in varying degrees of cyclopia, depending on the strength of the inhibitor and the exact time of its operation.

This analysis of the cause of cyclopia is interesting in the light of the present investigation, which has shown that the median region in early developmental stages of chick blastoderms has the greatest capacity for eye production. If certain physiological processes necessary for the production of bilateral eyes were inhibited, the result would be a morphological expression of the physiological condition in existence at the time of the inhibition; i. e., the production of a single median eye. Should an inhibitor be applied at a stage in which the eye-forming capacity is greatest in the lateral regions (during the process of the formation of the optic vesicles in the case of the chick), the result possibly would be a reduction in the size of the bilateral eyes, microphthalmia, and not cyclopia. Wright and Wagner (1934) have pointed out that an inhibitory process after the separation of the two optic primordia is probably the cause of microphthalmia.

SUMMARY

1. Grafts from implants from various regions of the pellucid area of embryos at late streak and head-process stages in development have been examined for eye production. In late primitive streak stages, an eye-forming area has been found which extends 0.06 mm. anterior, 0.02 mm. posterior to the primitive pit, and 0.2 mm. on each side of it. A corresponding area is located for head-process stages, extending 0.05 mm. anterior, and 0.1 mm. posterior to the anterior end of the head process, and 0.2 mm. on each side of it.

2. Both median and lateral regions within this area have the

capacity to produce the essential components of the eye cup, retina and pigmented layers, and a lens.

3. As a result of a comparative study of eye-forming frequency and type of differentiation in grafts from median and lateral regions, it seems apparent that there is a medio-lateral gradient in capacity for eye production, a greater capacity residing in left than in right regions.

4. During the developmental process a change occurs in the relative developmental capacities of median and lateral regions for eye production. As a consequence, at early somite stages the capacity of the lateral regions (primordia of optic vesicles) is greater than that of the median (primordium of the brain floor). The median region of the brain floor retains its potentiality for eye production up to the eight-somite stage, beyond which this potency is apparently lost.

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